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(54) Title: INHIBITORS OF HUMAN NEUTROPHIL ELASTASE AND HUMAN CATHEPSIN G

(57) Abstract

Novel small proteins which bind elastase or cathepsin G have been identified. These are useful as inhibitors of excessive elastase or cathepsin G activity in patients.

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Inhibitors of human Neutrophil Elastase and human Cathepsin G

BACKGROUND OF THE INVENTION

Field of the invention

The present invention relates to novel protease inhibitors and, in particular to small engineered proteins that inhibit human neutrophil elastase (hNE) and to proteins that inhibit human cathepsin G (hCG).

Description of the Background Art

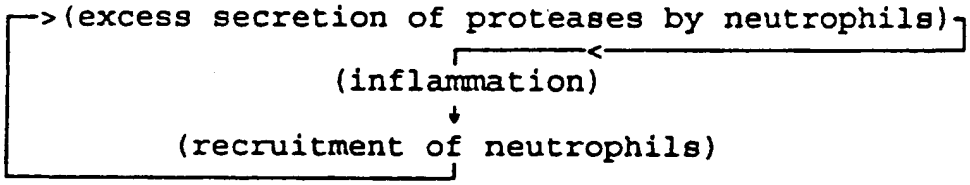
Neutrophil Elastase and Cathepsin G. The active sites of serine proteases are highly similar. Trypsin, chymotrypsin, neutrophil elastase, cathepsin G and many other proteases share strong sequence homology. The so-called catalytic triad comprises (in standard chymotrypsinogen numbering) aspartic acid-102, histidine-57, and serine-195. Residues close to the catalytic triad determine the substrate specificity of the particular enzyme (Cf. CREI84, p366-7). The structure and function of the digestive enzymes trypsin, pancreatic elastase, and chymotrypsin has been more throughly studied than have the neutrophil enzymes. X-ray structures of hNE complexed with a substrate have been solved and the similarity of the active site of hNE to that of trypsin is very high. The specificity of hNE is higher than trypsin and lower than Factor X_a.

Serine proteases are ubiquitous in living organisms and play vital roles in processes such as: digestion, blood clotting, fibrinolysis, immune response, fertilization, and post-translational processing of peptide hormones. Although the roles these enzymes play is vital, uncontrolled or inappropriate proteolytic activity can be very damaging. Several serine proteases are directly involved in serious disease states.

Human Neutrophil Elastase (hNE, or HLE; EC 3.4.21.11) is a 29 Kd serum protease with a wide spectrum of activity against extracellular matrix components (CAMP82, CAMP88, MCWH89, and references therein). The enzyme is one of the major neutral

proteases of the azurophil granules of polymorphonuclear leucocytes and is involved in the elimination of pathogens and in connective tissue restructuring (TRAV88). In cases of hereditary reduction of the circulating alpha-1-anti-protease inhibitor (α_1-PI), the principal physiological inhibitor of hNE (HEID86), or the inactivation of α_1-PI by oxidation ("smoker's emphysema"), extensive destruction of lung tissue may result from uncontrolled elastolytic activity of hNE (CANT89, BEIT86, HUBB86, HUBB89a,b, HUTC87, SOMM90, WEWE87). Several human respiratory disorders, including cystic fibrosis and emphysema, are characterized by an increased neutrophil burden on the epithelial surface of the lungs (SNID91, MCEL91, GOLD86) and hNE release by neutrophils is implicated in the progress of these disorders (MCEL91, WEIS89).

hNE is implicated as an essential ingredient in the pernicious cycle of:



observed in cystic fibrosis (CF) (NADE90). Inappropriate hNE activity is very harmful and to stop the progression of emphysema or to alleviate the symptoms of CF, an inhibitor of very high affinity is needed. The inhibitor must be very specific to hNE lest it inhibit other vital serine proteases or esterases. Nadel (NADE90) has suggested that onset of excess secretion is initiated by 10^{-10} M hNE; thus, the inhibitor must reduce the concentration of free hNE to well below this level. Thus, hNE is an enzyme for which an excellent inhibitor is needed.

There are reports that suggest that Proteinase 3 (also known as p29 or PR-3) is as important or even more important than hNE; see NILE89, ARNA90, KAOR88, CAMP90, and GUPT90. Cathepsin G is another protease produced by neutrophils that may cause disease when present in excess; see FERR90, PETE89, SALV87, and SOMM90.

Cathepsin G is less stable than hNE and thus harder to study *in vitro*. Powers and Harper (POWE86) indicate that cathepsin G is involved in inflammation, emphysema, adult respiratory distress syndrome, and rheumatoid arthritis.

Proteinaceous Serine Protease Inhibitors. A large number of proteins act as serine protease inhibitors by serving as a highly specific, limited proteolysis substrate for their target enzymes. In many cases, the reactive site peptide bond ("scissile bond") is encompassed in at least one disulfide loop, which insures that during conversion of virgin to modified inhibitor the two peptide chains cannot dissociate.

A special nomenclature has evolved for describing the active site of the inhibitor. Starting at the residue on the amino side of the scissile bond, and moving away from the bond, residues are named P1, P2, P3, etc. (SCHE67). Residues that follow the scissile bond are called P1', P2', P3', etc. It has been found that the main chain of protein inhibitors having very different overall structure are highly similar in the region between P3 and P3' with especially high similarity for P2, P_1 and P1' (LASK80 and works cited therein). It is generally accepted that each serine protease has sites S1, S2, etc. that receive the side groups of residues P1, P2, etc. of the substrate or inhibitor and sites S1', S2', etc. that receive the side groups of P1', P2', etc. of the substrate or inhibitor (SCHE67). It is the interactions between the S sites and the P side groups that give the protease specificity with respect to substrates and the inhibitors specificity with respect to proteases.

The serine protease inhibitors have been grouped into families according to both sequence similarity and the topological relationship of their active site and disulfide loops. The families include the bovine pancreatic trypsin inhibitor (Kunitz), pancreatic secretory trypsin inhibitor (Kazal), the Bowman-Birk inhibitor, and soybean trypsin inhibitor (Kunitz) families. Some inhibitors have several reactive sites on a single polypeptide

chains, and these distinct domains may have different sequences, specificities, and even topologies.

One of the more unusual characteristics of these inhibitors is their ability to retain some form of inhibitory activity even after replacement of the P1 residue. It has further been found that substituting amino acids in the P_3 to P_3' region, and more particularly the P3 to P3' region, can greatly influence the specificity of an inhibitor. LASK80 suggested that among the BPTI (Kunitz) family, inhibitors with P1 Lys and Arg tend to inhibit trypsin, those with P1=Tyr, Phe, Trp, Leu and Met tend to inhibit chymotrypsin, and those with P1=Ala or Ser are likely to inhibit elastase. Among the Kazal inhibitors, they continue, inhibitors with P1 = Leu or Met are strong inhibitors of elastase, and in the Bowman-Kirk family elastase is inhibited with P1 Ala, but not with P1 Leu.

We will next discuss a number of proteinaceous anti-elastase and anti-cathepsin G inhibitors of particular interest. Known HNE and cathepsin G inhibitors include the Kunitz family inhibitor UTI (GEBH86), the eglin/barley family inhibitor eglin (SCHN86b), and the serpin family inhibitors alpha1-antichymotrypsin and alpha1-antitrypsin (BARR86).

α_1-Proteinase Inhibitor (α1-antitrypsin). A logical approach to treatment of diseases attributable to excessive hNE levels is treatment with the endogenous irreversible inhibitor, α_1-PI. STON90 reports on studies of the efficacy of α1-PI in protecting hamster lung from damage by hNE. They conclude that α1-PI is only about 16% as effective *in vivo* as one might have estimated from *in vitro* measurements. Nevertheless, α1-PI shows a therapeutic effect. A preliminary study of aerosol administration of α_1-PI to cystic fibrosis patients indicates that such treatment can be effective both in prevention of respiratory tissue damage and in augmentation of host antimicrobial defenses (MCEL91).

However, there are practical problems with its routine use as a pulmonary anti-elastolytic agent. These include the relatively

large size of the molecule (394 residues, 51 Kd), the lack of intramolecular stabilizing disulfide bridges, and specific post translational modifications of the protein by glycosylation at three sites. HEIM91 reports inhibition of PMN leukocyte-mediated endothelial cell detachment by protease inhibitors. They compared secretory leukocyte protease inhibitor (SLPI), α1-protease inhibitor (α1-PI), and a chloromethylketone inhibitor (CMK). While SLPI and CMK inhibited the hNE mediated cell detachment, α1-PI did not; the author suggest that, because of its size, α1-PI can not penetrate to the site at which hNE acts. As the inhibitors disclosed in the present invention are smaller than SLPI, we expect them to move freely throughout the extracellular space.

Moreover, both cleaved α_1-PI and the α_1-PI/hNE complex (BAND88a,b) may be neutrophil chemoattractants. This could be a serious disadvantage if one wishes to interupt the cycle by which excessive numbers of neutrophils migrate to the lung, release hNE, the hNE reacts with α_1-PI generating a signal for more neutrophils to migrate to the lungs. Hence a small, stable, nontoxic, and potent inhibitor of hNE would be of great therapeutic value.

Human Pancreatic Secretory Trypsin Inhibitor. This is a Kazal family inhibitor. The inhibitors of this family are stored in zymogen granules and secreted with the zymogens in pancreatic juice. In general, the natural Kazal inhibitors are specific for trypsin. However, there are exceptions, such as certain domains of ovomucoids and ovoinhibitors, that inhibit chymotrypsin, subtilisin and elastase.

While the wild type hPSTI is completely inactive toward hNE, Collins *et al.* (COLL90) report designed variants of human pancreatic secretory trypsin inhibitor (hPSTI) having high affinity for hNE. Three of the reported variants have K_i for hNE below 10 pM: PSTI-5D36 at 7.3 pM, PSTI-4A40 at 7 pM, and PSTI-4F21 at 5.2 pM.

Squash Seed Inhibitor. The squash seed inhibitors are yet

another family of serine protease inhibitors. Those reported so far have lysine or arginine at the P1 residue, inhibit trypsin, and are completely inactive toward hNE. McWherter *et al.* (1989) synthesized several homologues of the squash-seed inhibitor, CMTI-III. CMTI-III has a K_i for trypsin of $\sim 1.5 \cdot 10^{-12}$ M. McWherter *et al.* (MCWH89) suggested substitution of "moderately bulky hydrophobic groups" at P1 to confer HLE (same as hNE) specificity. For cathepsin G, they expected bulky (especially aromatic) side groups to be strongly preferred. They found that PHE, LEU, MET, and ALA were functional by their criteria; they did not test TRP, TYR, or HIS. (Note that ALA has the second <u>smallest</u> side group available.) They found that a wider set of substituted residues (VAL, ILE, LEU, ALA, PHE, MET, and GLY) gave detectable binding to HLE. In particular, CMTI-III(VAL_5) has K_i = 9 nM relative to hNE.

"Kunitz" Domain Proteinase Inhibitors. Bovine pancreatic trypsin inibitor (BPTI, a.k.a. aprotonin) is a 58 a.a. serine proteinase inhibitor of the BPTI (Kunitz) domain (KuDom) family. Under the tradename TRASYLOL, it is used for countering the effects of trypsin released during pancreatitis. Not only is its 58 amino acid sequence known, the 3D structure of BPTI has been determined at high resolution by X-ray diffraction (HUBE77, MARQ83, WLOD84, WLOD87a, WLOD87b), neutron diffraction (WLOD84), and by NMR (WAGN87). One of the X-ray structures is deposited in the Brookhaven Protein Data Bank as "6PTI" [sic]. The 3D structure of various BPTI homologues (EIGE90, HYNE90) are also known. At least sixty homologues have been reported; the sequences of 39 homologues are given in Table 13, and the amino acid types appearing at each position are compiled in Table 15. The known human homologues include domains of Lipoprotein Associated Coagulation Inhibitor (LACI) (WUNT88, GIRA89), Inter-α-Trypsin Inhibitor (ALBR83a, ALBR83b, DIAR90, ENGH89, TRIB86, GEBH86, GEBH90, KAUM86, ODOM90, SALI90), and the Alzheimer beta-Amyloid Precursor Protein. Circularized BPTI and circularly permuted BPTI have binding properties similar to BPTI (GOLD83). Some

proteins homologous to BPTI have more or fewer residues at either terminus.

In BPTI, the P1 residue is at position 15. Tschesche *et al.* (TSCH87) reported on the binding of several BPTI P1 derivatives to various proteases:

Dissociation constants for BPTI P1 derivatives, Molar.

Residue #15 P1	Trypsin (bovine pancreas)	Chymotrypsin (bovine pancreas)	Elastase (porcine pancreas)	Elastase (human leukocytes)
lysine	$6.0 \cdot 10^{-14}$	$9.0 \cdot 10^{-9}$	-	$3.5 \cdot 10^{-6}$ (WT)
glycine	-	-	+	$7.0 \cdot 10^{-9}$
alanine	+	-	$2.8 \cdot 10^{-8}$	$2.5 \cdot 10^{-9}$
valine	-	-	$5.7 \cdot 10^{-8}$	$1.1 \cdot 10^{-10}$
leucine	-	-	$1.9 \cdot 10^{-8}$	$2.9 \cdot 10^{-9}$

From the report of Tschesche *et al.* we infer that molecular pairs marked "+" have K_ds $\geq 3.5 \cdot 10^{-6}$ M and that molecular pairs marked "-" have K_ds >> $3.5 \cdot 10^{-6}$ M. It is apparent that wild-type BPTI has only modest affinity for hNE, however, mutants of BPTI with higher affinity are known. While not shown in the Table, BPTI does not significantly bind hCG. However, Brinkmann and Tschesche (BRIN90) made a triple mutant of BPTI (*viz.* K15F, R17F, M52E) that has a K_i with respect to hCG of 5.0×10^{-7} M.

Works concerning BPTI and its homologues include: STAT87, SCHW87, GOLD83, CHAZ83, CREI74, CREI77a, CREI77b, CREI80, SIEK87, SINH90, RUEH73, HUBE74, HUBE75, HUBE77KIDO88, PONT88, KIDO90, AUER87, AUER90, SCOT87b, AUER88, AUER89, BECK88b, WACH79, WACH80, BECK89a, DUFT85, FIOR88, GIRA89, GOLD84, GOLD88, HOCH84, RITO83, NORR89a, NORR89b, OLTE89, SWAI88, and WAGN79.

Inter-α-trypsin inhibitor (ITI) is a large (M_r *ca* 240,000) circulating protease inhibitor found in the plasma of many mammalian species (for reviews see ODOM90, SALI90, GEBH90, GEBH86). Its affinity constant for hNE is 60-150 nM; for Cathepsin G it is 20-6000 nM. The intact inhibitor is a glyco-

protein and is currently believed to consist of three glycosylated subunits that interact through a strong glycosaminoglycan linkage (ODOM90, SALI90, ENGH89, SELL87). The anti-trypsin activity of ITI is located on the smallest subunit (ITI light chain, unglycosylated M_r *ca* 15,000) which is identical in amino acid sequence to an acid stable inhibitor found in urine (UTI) and serum (STI) (GEBH86, GEBH90). The mature light chain consists of a 21 residue N-terminal sequence, glycosylated at SER_{10}, followed by two tandem KuDoms the first of which is glycosylated at ASN_{45} (ODOM90). In the human protein, the second KuDom (ITI-D2 or HI-8t) has been shown to inhibit trypsin, chymotrypsin, and plasmin (ALBR83a, ALBR83b, SELL87, SWAI88). The first domain (ITI-D1 or HI-8e, comprising residues 22-76 of the UTI sequence shown in Fig. 1 of GEBH86) lacks these activities (ALBR83a,n, SWAI88) but has been reported to inhibit leukocyte elastase ($10^{-6} > K_i > 10^{-9}$) (-ALBR83a,b, ODOM90) and cathepsin G (SWAI88, ODOM90). The affinity is, however, too weak to be directly useful.

Sinha *et al.* (SINH91) report converting the KuDom of Alzheimer's β-amyloid precursor protein into an hNE inhibitor having K_i = 800 pM when valine was substituted for arginine at the P1 site (residue 13). They made a second protein having three mutations (*viz.* R13V(P1), A14S(P1'), M15I(P2')). The changes at P1' and P2' correspond to the amino acids found in the active site of α_1-PI. This protein is completely inactive with respect to hNE. They state, "Caution should therefore be used in extrapolating site-specific mutagenesis results among mechanistically unrelated inhibitors. In addition, unpredictable results can be obtained even within the KuDom family, as our experience with chymotrypsin and kallikrein illustrate."

Nonproteinaceous Elastase Inhibitors. The compounds ICI 200,355 (SOMM91) and ICI 200,880 show strong preference for HNE over other proteases such as trypsin. These compounds are analogues of peptides in which the amide nitrogen of the scissile bond has been replaced by a CF_3 group. Each of these compounds

have an isopropyl group (as does valine) at the P1 position and a prolyl residue at P2. Neither compound has any extension toward P1'. Imperiali and Abeles (IMPE86) describe protease inhibitors consisting of acetyl peptidyl methyl ketones in which the terminal methyl group bears zero to three fluorine atoms; there is no P1' residue in any of their compounds. PEET90 (and works cited therein) report synthesis of peptidyl fluoromethyl ketones and peptidyl α-keto esters and the inhibitory properties of these compounds relative to porcine pancreatic elastase (PPE), HNE, rat cathepsin G, and human cathepsin G; these compounds do not extend to P1'. Mehdi et al. (MEHD90) report inhibition of HNE and human cathepsin G by methyl esters of peptidyl α-keto carboxylic acids; none of these compounds contain P1' residues. Angelastro et al. (ANGE90) report protease inhibitors having diketo groups; none of these compounds extend beyond P1.

Govhardan and Abeles (GOVH90) describe compounds in which the amide -NH- has been replaced by $-CF_2-CH_2-$ followed by an α-amino-linked amino-acid methyl ester, thus providing a P1' residue.

Imperiali and Abeles (IMPR87) describe inhibitors of chymotrypsin extending to P3'. Works cited by these authors indicate that the inhibitory constant, K_i, can be lowered by specifically matching the S1', S2', S3', ... binding sites on the protease. These authors do not discuss inhibition of HNE. Furthermore, their inhibitors are not derived from high affinity protein protease inhibitors; rather the side groups at P1', P2', and P3' are determined by trial and error. In addition, between P1 and P1', they insert $-CO-CF_2-CH_2-$ in place of -CO-NH- so that the distal part of the chain is displaced. We prefer to replace -CO-NH- with $-CO-CF_2-$ or -CO-CFH- so that the remainder of the residues can take up conformations highly similar to those found in EpiNE proteins.

Another class of protease inhibitors are those in which the carbonyl carbon of the scissile peptide is replaced by boron. These compounds inhibit serine proteases, but are not very

specific.

Another class of elastase inhibitors are the chloromethylketones as described by Robert et al. (US 4,665,053). These compounds have a chlorine atom adjacent to a keto group. The active-site serine of the protease acts as a nucleophile, displacing chloride and yielding a covalent enzyme-inhibitor adduct that is irreversibly inactive. An-Zhi et al. (FEBS Lett. 234 (2) 367-373 (1988)) describe the X-ray crystal structure of HNE with a peptidyl chloromethyl ketone. Tsuda et al. (Chem Pharm Bull, 35(9)3576-84 (1987)) describe synthesis of peptide chloromethyl ketones and their activity against proteases, including HNE. Ganu and Shaw (Thrombosis Research, 45:1-6 (1987)) describe improved peptidyl chloromethyl ketone plasmin inhibitors. Because the chloromethyl ketones form irreversible adducts, they are less desirable as drugs. Other classes of inhibitors that form irreversible complexes include a) peptide enol lactones (J Biol Chem 266(1)13-21 (1991) and Biochemistry 29:4305-11 (1990)), isocoumarins (Krantz et al., US 4,657,893, Powers et al., US 4,845,242, and Kobuko et al., US 4,980287), and peptidyl (α-aminoalkyl)phosphonate diphenyl esters (Biochemistry 30:485-93 (1991)).

A class of compounds, related to the chloromethyl ketones, that bind reversibly to proteases with some degree of specificity comprises peptidyl methyl ketones. Peters and Fittkau (Biomed Biochim Acta 49(4)173-178 (1990) and works cited therein) report that peptidyl methyl ketones bind serine- and cysteine-proteases reversibly and that the binding depends on the sequence of the peptidyl group. If the peptidyl methyl ketones are viewed as peptide analogues in which the carbonyl group of an amino acid is replaced by a methyl group, Peters and Fittkau discuss only compounds that are extended toward the amino terminus. Thus, they supply P1, P2, etc., but not P1', P2', etc.

Miscellaneous Information on Elastase Inhibition. PADR91 reports that elastin (the definitive substrate of all elastases) greatly reduces the efficacy of a variety of reversible and

irreversible hNE inhibitors when compared to the efficacy determined with small, soluble artificial substrates. They found that both classes of inhibitors have from 20-fold to more than 100-fold less efficacy. They suggest that elastin reduces the on rate, but say they have no explanation for this phenomenon. One possibility is that the synthetic inhibitors (all rather hydrophobic) bind to elastin (which is also hydrophobic). They tested one reversible protein inhibitor, mucus protease inhibitor, which has K_i = 30 nM without elastin or 900 nM with elastin. If our inhibitors suffer a 30-fold loss of efficacy, they can still reduce free hNE to below 10^{-10} M.

No admission is made that any cited reference is prior art or pertinent prior art, and the dates given are those appearing on the reference and may not be identical to the actual publication date. All references cited in this specification are hereby incorporated by reference.

SUMMARY OF THE INVENTION

The present invention relates to mutants of Kunitz Domain serine protease inhibitors, such as BPTI and ITI-D1, with substantially enhanced affinity for elastase. These muteins have an affinity for elastase estimated to be at least an order of magnitude higher than that of the wild-type domain and, in some instances, at least three orders of magnitude (1000-fold) higher.

For some of the proteins, kinetic inhibitory data show that the binding affinity is in the range 1.0×10^{-12} M to 3.0×10^{-12} M. Other proteins are displayed on fusion phage and the affinity for hNE or hCG is estimated by the pH elution profile from immobilized active protease (hNE or hCG). A number of the proteins have been produced in useful quantities as secreted proteins in yeast.

The present invention also relates to linear and cyclic oligopeptide analogues of aprotonin and related polypeptides that specifically bind human neutrophil elastase and/or cathepsin G. It relates in particular to analogues of the novel elastase-

binding polypeptides (EpiNe) and cathepsin G-binding polypeptides disclosed herein.

These analogues differ from aprotonin and its kindred inhibitors in several respects. First, they are smaller molecules, preferably less than 1,500 daltons molecular weight, and including only the P_3-P_3' residues (or analogues thereof) or a subset thereof. Secondly, the scissile peptide (-CO-NH-) bond between the P_1 and P_1' residues is replaced by a substantially nonhydrolyzable bond that substantially maintains the distance between the alpha carbons of those two residues.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates fractionation of the Mini PEPI library on hNE beads. The abscissae shows pH of buffer. The ordinants show amount of phage (as fraction of input phage) obtained at given pH. Ordinants scaled by 10^3.

Figure 2 illustrates fractionation of the MYMUT PEPI library on hNE beads. The abscissae shows pH of buffer. The ordinants show amount of phage (as fraction of input phage) obtained at given pH. Ordinants scaled by 10^3.

Figure 3 shows the elution profiles for EpiNE clones 1, 3, and 7. Each profile is scaled so that the peak is 1.0 to emphasize the shape of the curve.

Figure 4 shows pH profile for the binding of BPTI-III MK and EpiNE1 on cathepsin G beads. The abscissae shows pH of buffer. The ordinants show amount of phage (as fraction of input phage) obtained at given pH. Ordinants scaled by 10^3.

Figure 5 shows pH profile for the fractionation of the MYMUT Library on cathepsin G beads. The abscissae shows pH of buffer. The ordinants show amount of phage (as fraction of input phage) obtained at given pH. Ordinants scaled by 10^3.

Figure 6 shows a second fractionation of MYMUT library over cathepsin G.

Figure 7 shows elution profiles on immobilized cathepsin G for

phage selected for binding to cathepsin G.

Figure 8 shows the form of one group of preferred HNE inhibitors, hereinafter Class I inhibitors. Carbons marked 7, 8, 9, and 10 are chiral centers.

Figure 9 shows the form of a second group of preferred HNE inhibitors, hereinafter Class II inhibitors. Carbons marked 7, 8, 9, and 10 are chiral centers.

Figure 10 shows 2-carboxymethyl-6-aminomethyl anthraquinone as a linker. Other relatively rigid molecules of similar dimension can be used.

Figure 11 shows compounds I through XVIII involved in preparing analogues of the VAL-ALA dipeptide having -NH- replaced by $-CF_2-$, $-CH_2-$, or -CHF- for Class I and Class II inhibitors.

Figure 12 shows the form of a third group of preferred HNE inhibitors, hereinafter Class III inhibitors. Carbons marked 8, 9 and 10 are chiral centers.

Figure 13 shows compounds XXXI through XXXV that are involved in synthesizing the boron-containing dipeptide analogue used in Class I and Class II inhibitors.

Figure 14 shows compounds XLI through XLIV that are involved in synthesis of a portion of the molecule shown in Figure 5.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Small Proteins With High Affinity for Elastase or Cathepsin G

The present invention relates to muteins of BPTI, ITI-D1 and other Kunitz domain-type inhibitors which have a high affinity for elastase and cathepsin G. Some of the described inhibitors are derived from BPTI and some from ITI-D1. However, hybrids of the identified muteins and other Kunitz domain-type inhibitors could be constructed.

For the purpose of simultaneously assessing the affinity of a large number of different BPTI and ITI-D1 muteins, DNA sequences encoding the BPTI or ITI-DI was incorporated into the genome of

the bacteriophage M13. The KuDom is displayed on the surface of M13 as an amino-terminal fusion with the gene III coat protein. Alterations in the KuDom amino acid sequence were introduced. Each pure population of phage displaying a particular KuDom was characterized with regard to its interactions with immobilized hNE or hCG. Based on comparison to the pH elution profiles of phage displaying other KuDoms of known affinities for the particular protease, mutant KuDoms having high affinity for the target proteases were identified. Subsequently, the sequences of these mutant KuDoms were determined (typically by sequencing the corresponding DNA sequence).

Certain aprotonin-like protease inhibitors were shown to have a high affinity for HNE ($\sim 10^{12}$/M). These 58 amino acid polypeptides were biologically selected from a library of aprotinin mutants produced through synthetic diversity. Positions P1, P1', P2', P3', and P4' were varied. At P1, only VAL and ILE were selected, although LEU, PHE, and MET were allowed by the synthetic conditions. At P1', ALA and GLY were allowed and both were found in proteins having high affinity. (While not explored in the library, many Kazal family inhibitors of serine proteases have glutamic or aspartic acid at P1'.) All selected proteins contained either PHE or MET at P2'; LEU, ILE, and VAL, which are amino acids with branched aliphatic side groups, were in the library but apparently hinder binding to HNE. Surprisingly, position P3' of all proteins selected for high affinity for HNE have phenylalanine. No one had suggested that P3' was a crucial position for determining specificity relative to HNE. At P4', SER, PRO, THR, LYS, and GLN were allowed; all of these except THR were observed. PRO and SER are found in the derivatives having the highest affinity.

As previously noted, BPTI is a protein of 58 amino acids. The sequence of BPTI is given in entry 1 of Table 13. The invention is not limited to 58-amino-acid proteins, as homologues having more or fewer amino acids are expected to be active.

Wild-type BPTI is not a good inhibitor of hNE. BPTI with a single K15L mutation exhibits a moderate affinity for HNE (K_d = $2.9 \cdot 10^{-9}$ M) (BECK88b). However, the amino terminal Kunitz domain (BI-8e) of the light chain of bovine inter-α-trypsin inhibitor has been generated by proteolysis and shown to be a potent inhibitor of HNE (K_d = $4.4 \cdot 10^{-11}$ M) (ALBR83).

It has been proposed that the P1 residue is the primary determinant of the specificity and potency of BPTI-like molecules (SINH91, BECK88b, LASK80 and works cited therein). Although both BI-8e and BPTI(K15L) feature LEU at their respective P1 positions, there is a 66 fold difference in the affinities of these molecules for HNE. We therefore hypothesized that other structural features must contribute to the affinity of BPTI-like molecules for HNE.

A comparison of the structures of BI-8e and BPTI(K15L) reveals the presence of three positively charged residues at positions 39, 41, and 42 of BPTI which are absent in BI-8e. These hydrophilic and highly charged residues of BPTI are displayed on a loop which underlies the loop containing the P1 residue and is connected to it *via* a disulfide bridge. Residues within the underlying loop (in particular residue 39) participate in the interaction of BPTI with the surface of trypsin (BLOW72) and may contribute significantly to the tenacious binding of BPTI to trypsin. These hydrophilic residues might, however, hamper the docking of BPTI variants with HNE. Supporting this hypothesis, BI-8e displays a high affinity for HNE and contains no charged residues in residues 39-42. Hence, residues 39 through 42 of wild type BPTI were replaced with the corresponding residues (MGNG) of the human homologue of BI-8e. As we anticipated, a BPTI(K15L) derivative containing the MGNG 39-42 substitution exhibited a higher affinity for HNE than did the single substitution mutant BPTI(K15L). Mutants of BPTI with Met at position 39 are known, but positions 40-42 were not mutated simultaneously.

Tables 207 and 208 present the sequences of additional novel BPTI mutants with high affinity for hNE. We believe these mutants

to have an affinity for hNE which is about an order of magnitude higher than that of BPTI (K15V, R17L). All of these mutants contain, besides the active site mutations shown in the Tables, the MGNG mutation at positions 39-42.

Similarly, Table 209 presents the sequences of novel BPTI mutants with high affinity for cathepsin G. The P1 residue in the EpiC mutants is predominantly MET, with one example of PHE, while in BPTI P1 is LYS and in the EpiNE variants P1 is either VAL or ILE. In the EpiC mutants, P1' (residue 16) is predominantly ALA with one example of GLY and P2' (residue 17) is PHE, ILE, or LEU. Interestingly, residues 16 and 17 appear to pair off by complementary size, at least in this small sample. The small GLY residue pairs with the bulky PHE while the relatively larger ALA residue pairs with the less bulky LEU and ILE. Alternatively, the pairing could be according to flexibility at P1'; glycine at P1' might allow the side group of phenylalanine to reach a pocket that is not accessible when P1' is alanine. When P1' is alanine, leucine or isoleucine appear to be the best choice.

Although BPTI has been used in humans with very few adverse effects, a KuDom having much higher similarity to a human KuDom poses much less risk of causing an immune response. Thus, we transferred the active site changes found in EpiNE7 into the first KuDom of inter-α-trypsin inhibitor (Example IV). For the purpose of this application, the numbering of the nucleic acid sequence for the ITI light chain gene is that of TRAB86 and that of the amino acid sequence is the one shown for UTI in FIg. 1 of GEBH86. The necessary coding sequence for ITI-DI is the 168 bases between positions 750 and 917 in the cDNA sequence presented in TRAB86. The amino acid sequence of human ITI-D1 is 56 amino acids long, extending from Lys-22 to Arg-77 of the complete ITI light chain sequence. The P1 site of ITI-DI is Met-36. Tables 220-221 present certain ITI mutants; note that the residues are numbered according to the homologus Kunitz domain of BPTI, i.e., with the P1 residue numbered 15. It should be noted that it is probably acceptable to truncate the amino-terminal of ITI-D1, at least up to the first

residue homologous with BPTI.

The EpiNE7-inspired mutation (BPTI 15-19 region) of ITI-D1 significantly enhanced its affinity for hNE. We also discovered that mutation of a different part of the molecule (BPTI 1-4 region) provided a similar increase in affinity. When these two mutational patterns were combined, a synergistic increase in affinity was observed. Further mutations in nearby amino acids (BPTI 26, 31, 34) led to additional improvements in affinity.

The elastase-binding muteins of ITI-DI envisioned herein preferably differ from the wild-type domain at one or more of the following positions (numbered per BPTI): 1, 2, 4, 15, 16, 18, 19, 31 and 34. More preferably, they exhibit one or more of the following mutations: Lys1 -> Arg; Glu2 -> Pro; Ser4 -> Phe ; Met15 -> Val*, Ile; Gly16 -> Ala; THr18 -> Phe*; Ser19 -> Pro; Thr26 -> ALa; Glu31 -> Gln; Gln34 -> Val*. Introduction of one or more of the starred mutations is especially desirable, and, in one preferred embodiment, at least all of the starred mutations are present.

It will be recognized by those of ordinary skill in the art that the identified HNE and HCG inhibitors, may be modified in such a manner that the change will not greatly diminish the affinity, specificity, or stability of the inhibitor. Proposed changes can be assessed on several bases. First we ask whether a particular amino acid can fit into the KuDom framework at a given location; a change that disrupts the framework is very likely to impair binding and lower specificity. The likelihood that an amino acid can fit into the KuDom framework can be judged in several ways: 1) does the amino acid appear there in any known KuDom? 2) Do structural models of KuDoms indicate compatibility between the structure and the proposed substitution? and 3) do dynamic computational models suggest that the proposed mutant protein will be stable? The sequence variability of naturally-occurring KuDoms gives us proof that certain amino acids are acceptable at certain locations; lack of examples does not prove that the amino acid won't fit.

If a proposed change is deemed to be structurally acceptable, we then ask what effect it is likely to have on binding to the target and to other substances. Generally, a mutant protein having a changed residue in the interface between KuDom and target will need to be tested, usually *via* binding studies of a phage that displays the mutant protein. Most changes in the binding interface reduce binding, but some do increase affinity. Changes at residues far-removed from the binding interface usually do not reduce binding if the protein is not destabilized.

Table 61 shows the variability of 39 naturally-occurring Kunitz domains. All these proteins have 51 residues in the region C_5 through C_{55}; the total number of residues varies due to the proteins having more or fewer residues at the termini. Table 62 list the names of the proteins that are included in Table 61. Table 64 cites works where these sequences are recorded. Table 63 shows a histogram of how many loci show a particular variability *vs.* the variability. "Core" refers to residues from 5 to 55 that show greater sequence and structural similarity than do residues outside the core.

At ten positions a single amino-acid type is observed in all 42 cases, these are C_5, G_{12}, C_{14}, C_{30}, F_{33}, G_{37}, C_{38}, N_{43}, C_{51}, and C_{55}. Although there are reports that each of these positions may be substituted without complete loss of structure, only G_{12}, C_{14}, G_{37}, and C_{38} are close enough to the binding interface to offer any incentive to make changes. G_{12} is in a conformation that only glycine can attain; this residue is best left as is. Marks *et al.* (MARK87) replaced both C_{14} and C_{38} with either two alanines or two threonines. The C_{14}/C_{38} cystine bridge that Marks *et al.* removed is the one very close to the scissile bond in BPTI; surprisingly, both mutant molecules functioned as trypsin inhibitors. Both BPTI(C14A,C38A) and BPTI(C14T,C38T) are stable and inhibit trypsin. Altering these residues might give rise to a useful inhibitor that retains a useful stability, and the phage-display of a variegated population is the best way to obtain and test

mutants that embody alterations at either 14 or 38. Only if the C_{14}/C_{38} disulfide is removed, would the strict conservation of G_{37} be removed.

At seven positions (*viz.* 23, 35, 36, 40, 41, 45, and 47) only two amino-acid types have been found. At position 23 only Y and F are observed; the *para* position of the phenyl ring is solvent accessible and far from the binding site. Changes here are likely to exert subtle influences on binding and are not a high priority for variegation. Similarly, 35 has only the aromatic residues Y and W; phenylalanine would probably function well here. At 36, glycine predominates while serine is also seen. Other amino acids, especially {N, D, A, R}, should be allowed and would likely affect binding properties. Position 40 has only G or A; structural models suggest that other amino acids would be tolerated, particularly those in the set {S, D, N, E, K, R, L, M, Q, and T}. Position 40 is close enough to the binding site that alteration here might affect binding. At 41, only N, and K have been seen, but any amino acid, other than proline, should be allowed. The side group is exposed, so hydrophilic side groups are preferred, especially {D, S, T, E, R, Q, and A}. This residue is far enough from the binding site that changes here are not expected to have big effects on binding. At 45, F is highly preferred, but Y is observed once. As one edge of the phenyl ring is exposed, substitution of other aromatics (W or H) is likely to make molecules of similar structure, though it is difficult to predict how the stability will be affected. Aliphatics such as leucine or methionine (not having branched C_βs) might also work here. At 47, only S and T have been seen, but other amino acids, especially {N, D, G, and A}, should give stable proteins.

At one position (44), only three amino-acid types have been observed. Here, asparagine predominates and may form internal hydrogen bonds. Other amino acids should be allowed, excepting perhaps proline.

At the remaining 40 positions, four or more amino acids have

been observed; at 28 positions, eight or more amino-acid types are seen. Position 25 exhibits 13 different types and 5 positions (1, 6, 17, 26, and 34) exhibit 12 types. Proline (the most rigid amino acid) has been observed at fourteen positions: 1, 2, 8, 9, 11, 13, 19, 25, 32, 34, 39, 49, 57, and 58. The ϕ,ψ angles of BPTI (CREI84, Table 6-3, p. 222) indicate that proline should be allowed at positions 1, 2, 3, 7, 8, 9, 11, 13, 16, 19, 23, 25, 26, 32, 35, 36, 40, 42, 43, 48, 49, 50, 52, 53, 54, 56, and 58. Proline occurs at four positions (34, 39, 57, and 58) where the BPTI ϕ,ψ angles indicate that it should be unacceptable. We conclude that the main chain rearranges locally in these cases.

Based on these data and excluding the six cysteines, we judge that the KuDom structure will allow those substitutions shown in Table 65. The class indicates whether the substitutions: A) are very likely to give a stable protein having substantially the same binding to hNE, hCG, or some other serine protease as the parental sequence, B) are likely to give similar binding as the parent, or C) are likely to give a proteins retaining the KuDom structure, but which are likely to affect the binding. Mutants in class C must be tested for affinity, which is relatively easy using a display-phage system, such as the one set forth in WO/02809. The affinity of hNE and hCG inhibitors is most sensitive to substitutions at positions 15, 16, 17, 18, 34, 3[illegible], 19, 13, 11, 20, 36 of BPTI, if the inhibitor is a mutant of ITI-D1, these positions must be converted to their ITI-D1 equivalents by aligning the cysteines in BPTI and ITI-D1.

Certain of our hNE inhibitors will be useful as PR-3 inhibitors. We have modeled the interaction of our inhibitors with hNE by reference to the BPTI-trypsin complex. First we listed the residues of trypsin that touch BPTI. Next we considered the corresponding sets of residues from hNE and PR-3. These sets differ at eleven residues. Only one of the differences occurs in the S1 specificity pocket, viz. V_{190} in hNE vs. I_{190} in PR3. We therefore believe that our hNE inhibitors are likely to

be PR-3 inhibitors as well. In particular, the inhibitors having valine at P1 are likely to inhibit PR3. PR3 has an extra methyl in this region so the inhibitors having one fewer methyls are more likely to bind tightly.

BPTI is quite small; if this should cause a pharmacological problem, such as excessively quick elimination from the circulation, two or more BPTI-derived domains may be joined by a linker. This linker is preferably a sequence of one or more amino acids. A preferred linker is one found between repeated domains of a human protein, especially the linkers found in human BPTI homologues, one of which has two domains (BALD85, ALBR83b) and another of which three (WUNT88). Peptide linkers have the advantage that the entire protein may then be expressed by recombinant DNA techniques. It is also possible to use a nonpeptidyl linker, such as one of those commonly used to form immunogenic conjugates. For example, a BPTI-like KuDom to polyethyleneglycol, so called PEGylation (DAVI79).

Another possible pharmacological problem is immunigenicity. BPTI has been used in humans with very few adverse effects. Siekmann *et al.* (SIEK89) have studied immunological characteristics of BPTI and some homologues. Furthermore, one can reduce the probability of immune response by starting with a human protein. Thus, by changing nonessential residues, one may change the protein to more closely resemble a human protein. Other modifications, such as PEGylation, have also been shown to reduce immune responce (DAVI79).

Derivatized Peptides Which Bind Elastase or Cathepsin G

The present invention also relates to certain derivatized peptides which bind elastase or cathepsin G. The description which follows relates particularly to derivatization of EpiNE-type hNE inhibitors, but is applicable, mutatis mutandis, to EpiC-type cathepsin G inhibitors and ITID1-type hNE inhibitors as well. One embodiment consists of Class I inhibitors (shown in Figure 8), each of which comprise 1) a first segment of peptide residues, 2) an amino-acid analogue that binds to the S1 pocket of HNE but that

can not be hydrolysed, and 3) a second segment of peptide residues. These Class I inhibitors have the structure depicted in Figure 8.

The first and second peptide segments and the side group of the P1 amino-acid analogue are picked to foster high affinity for HNE and to increase specificity relative to other proteases. The group that links the first and second peptide segments is picked: 1) to prevent cleavage, 2) to allow reversible binding to the active site of HNE, and 3) to mimic the shape and charge distribution of the peptide group.

A second embodiment of the invention comprises Class II inhibitors which are cyclic compounds as shown in Figure 9. These compounds comprise: 1) a first peptide segment linked to, 2) an amino-acid analogue that binds to the S1 pocket of HNE but that can not be hydrolysed, 3) a second segment of peptide residues, and 4) a relatively rigid segment that connects the carboxy end of the second peptide segment to the amino end of the first peptide segment. This fourth segment is designed so that the segments 1-3 tend to exist in the conformation that binds HNE. The considerations for segments 1-3 are the same in both classes of compounds.

The inhibitors of Class II, as depicted in Figure 9, have at R_1 a relatively rigid bifunctional linker such as a tricyclic aromatic ring system having diametrically opposed functionalities one of which allows linkage to the amino group attached to C_7 and another that allows linkage to the carbonyl carbon labeled C_{11}, e.g. 2-carboxymethyl-6-aminomethyl anthraquinone (figure 10). The substituents

R_2, X, R_3, R4, R_5, and R_7 have the same possibilities as those set forth above for Class I.

A third embodiment of the invention consists of Class III inhibitors shown in Figure 12 having peptides or peptide analogues corresponding to residues P1', P2', P3', and (optionally) P4' (and P_5'). A boronic acid group or a boronic acid ester is positioned

so that it can fit into the "active site" of the enzyme.

Methods of synthesizing these compounds are known to those skilled in the art.

EpiNE1, 3 and 7 have molecular weights of about ~6 kd. It it is possible to provide compounds much smaller than EpiNE1, 3, or 7 that have high affinity for HNE. Although the P5-P4...P4'-P5' strand of the EpiNE proteins are not the only determinants of specificity, this strand, or a subsequence thereof, is likely to bind very tightly to HNE. A derivative in which the scissile peptide is modified so that it can not be hydrolyzed is likely to be a highly effective HNE inhibitor.

Many of the analogues of the present invention may be defined by the following formula:

$$P_N\text{-}P_1\text{-}P_1'\text{-}P_2'\text{-}P_3'\text{-}P_C$$

wherein P_N is

$T\text{-}P_5\text{-}P_4\text{-}P_3\text{-}P_2\text{-}$,

$T\text{-}P_4\text{-}P_3\text{-}P_2\text{-}$,

$T\text{-}P_3\text{-}P_2\text{-}$,

$T\text{-}P_2\text{-}$, or

$T\text{-}$;

and wherein P_C is

$-P_4'\text{-}P_5'\text{-}T$,

$-P_4'\text{-}T$, or

$-T$;

and where P_5, P_4, P_3, and P_2, and P_2', P_3', P_4', and P_5', are amino acids, including but not necessarily limited to naturally occurring amino acids, which can serve the same function as the corresponding active site amino acids of the EpiNE polypeptides, and T is a termination functional group compatible with peptide synthesis and not adverse to elastase inhibitory activity of the peptide (the two Ts may be the same or different and may join to

form a cyclic structure);

and where either (1) P_1 is a residue of an amino acid analogue having the general formula -NH-CHR-X_C-, P_1' is a residue of an amino acid analogue having the general formula -X_N-CHR-CO-, P_1 and P_1' together forming -X_C-X_N-, which contains a nonhydrolyzable bond; or (2) P_1 and P'' together form a nonhydrolyzable boron-containing analogue of a dipeptide; in either case the P_1 and P_1' performing the same function as the corresponding amino acids of the EpiNE polypeptides.

Others have described a number of linkages that have dimensions quite similar to peptides but which can not be hydrolyzed. Most have provided only the residues P5-P4..P1 and modified P1 so that it binds the protease, reversibly or irreversibly. Their approach is flawed in several respects. First, they have not recognized the significance of the P_1'-P_3' residues. Second, they have not preserved the dimensions of the scissile bond of aprotonin.

Figure 8 shows Class I inhibitors, which are linear peptide analogues of the EpiNE polypeptides. Preferred choices for R_1 (P2), R_2 (P1), X, R_3 (P1'), R_4 (P2'), R_5 (P3'), and R_6 (P4') are as follows:

R_1 : H-, acetyl-, or a hydrophobic moiety, such as L-prolyl-, L,L cystinyl-, L-valyl-, L-methionyl-, and acetyl-. Note that R1 is the side group of what in aprotonin is the P2 residue, and that the inhibitor optionally may include the P3, P3-P4, or P3-P5 residues of aprotonin and its analogues, including the EpiNE polypeptides.

R_2 : alkyl or 2-4 carbon atoms, i.e., ethyl, n-propyl, isopropyl, n-butyl, isobutyl or tert-butyl. 2-propyl (so that C_γ resembles the C_α of L-Valine), and 2-butyl (so that C_γ resembles the C_α of L-Isoleucine) are especially preferred.

X : a nonhydrolyzable linker which does not interfere with

elastase-inhibitory activity. This linker preferably has a length similar to that of a peptide (-CO-NH-) group. If this linker is characterized as $-X_N-X_C-$, then $-X_N-$ may be -CO-, -SO- or $-B(OR_7)-$, and $-X_C-$ may be a thioether (-S-) or a methylene which is unsubstituted ($-CH_2-$), or which is mono- (e.g., -CHF) or di- (e.g., $-CF_2-$) substituted. The substituents may be methyl, ethyl, n-propyl, isopropyl, chlorine or fluorine, though it is preferable that no more than one substituent be halogen. Suitable linkers include $-CO-CH_2-$, $-CO-CF_2-$, -CO-CHF-, -CO-CO-, $-B(OH)-CH_2-$, $-B(OR_7)-CH_2-$, $-SO-CH_2-$, and -CO-S-.

The distance between the C-alpha carbons connected by a typical -CO-NH- peptidyl linkage is about 3.8 angstroms. The preferred nonpeptidyl, nonhydrolyzable linkages of the present invention do not increase the alpha-to-alpha distance to more than about 4.5 angstroms.

However, a longer linker, such as $-CO-CFH-CH_2-$ or $-CO-CF_2-CH_2-$, may be used. With these linkers, the alpha-to-alpha distance is increased to about 5-6 angstroms.

It is desirable, but not required, that the main atoms of the linker and the connected Calpha carbons lie substantially in the same plane, as is true for the normal peptide linkage.

R_3 : -H, or an aliphatic group containing 1-10 carbons and 0-3 N, O, S, Cl or F atoms, such as a small alkyl or alkoxy group. The functionalities -H, $-CH_3$, $-CH_2-COOH$, and $-CH_2-CH_2-COOH$, so that C_α resembles the Calpha of Glycine, L-Alanine, L-Aspartic Acid, or L-Glutamic Acid, respectively, are especially preferred. Other possibilities include ethyl, isopropyl, n-propyl, hydroxymethyl (i.e.,

forming serine), or hydroxyethyl (i.e., forming homoserine).

R_4 : an unbranched aliphatic group of 4-7 carbons, or an arylalkyl group, wherein the alkyl moiety is 1-3 carbons and the aryl moiety is monocyclic or bicyclic, and may contain heteroatoms (N,O,S) and may contain halogen (Cl,F) substitutions. The functionalities $-CH_2$-phenyl and $-CH_2-CH_2-S-CH_3$, so that C_9 resembles the Calpha of L-Phenylalanine or L-Methionine, respectively, are especially preferred.

R_5 : an arylalkyl group, as discussed under R4 above. More preferably, an arylmethyl group, especially the $-CH_2$-phenyl group (as in L-Phenylalanine).

R6 : $-NH_2$, -OH, or -AA where AA is an amino acid residue, such as serine, proline, or lysine, or a short peptide. The amino acid may be any amino acid found in the P_4' position of a BPTI (Kunitz) family inhibitor, including the EpiNE inhibitors. The short peptide is preferably the dipeptide sequence $P_4'-P_5'$ of such an inhibitor.

R7 : a small alkyl group (1-4 carbon atoms), such as $-CH_3$, $-CH_2-CH_3$, or $-CH(CH_3)_2$.

At R_1, it is preferable to have an unblocked amino group to improve solubility. The amino acid is preferably hydrophobic since, in the Epi molecules, there is a half-cystine at this position, and the disulfide (-S-S-) bond is hydrophobic.

At R_2, the 2-propyl group is especially preferred because EpiNE1 binds more tightly to HNE than do derivatives having ILE at P1. When R_2 is 2-butyl, the chirality at the ß carbon is, preferably, the same as in L-ILE found in nature. It is preferred that the carbon marked C_7 in Figure 1 have the same chirality as L-valine. Compounds having unspecified chirality at C_7 and C_8 may be usable. It is preferred that the chirality at C_9 and C_{10} be the same as L amino acids. R_2 could also be $-CH_3$, $-CF_3$, or $-CH_2-CH_3$.

At R_3, $-CH_3$ is preferred; -H, $-CH_2-COOH$, or $-CH_2OH$ may also be

used.

R_4 = $-CH_2$-phenyl is especially preferred, R_4 = $-CH_2-CH_2-S-CH_3$ is also preferred.

R_5 = $-CH_2$-phenyl is especially preferred. Other neutral aryl groups can be attached to the $-CH_2-$ group, such as mono- and dimethylphenyls, naphthyl (α or ß), hydroxyphenyl (o, m, or p), and methoxyphenyl (o, m, or p).

R_6 is picked for specificity and solubility; -OH, $-NH_2$, L-serine, L-proline, and L-lysine are preferred.

Synthesis of perfluoro compounds is often easier than is synthesis of compounds having some hydrogens and some fluorines. Thus X = [$-CO-CF_2-$], R_3 = -F or $-CF_3$, and replacing H_8 with F leads to a preferred compound.

Figure 9 shows Class II inhibitors, which are cyclic peptide analogues of the EpiNE compounds. Preferred choices for R_2, X, R_3, R_4, and R_5 are the same as for the Class I inhibitors (There is no R6). R_1 forms a bridge between the amide of the P1 residue and the carbonyl of the P_3' residue. It is a relatively rigid group having functional groups that allow the carbonyl carbon labeled C_{11} to link to one end of R_1 while a second functional group of R_1 can be linked to the amino group N_7. Functional groups which contain one or more rings help to impart the desired rigidity.

R_1 should also have the desired span, so that R_1 will hold C_7, C_8, C_9, and C_{10} in the appropriate conformation. In BPTI, C_α-15 and C-18 are separated by 10 Å. Therefore, R_1 should likewise provide a spacing of about 10 Å.

For example, in 2,6-dimethylanthracene, the methyl carbons are separated by about 9 Å. Because dimethylanthracene is shorter than the desired separation between C_α-15 and C-18, we attach a carboxylic acid to one methyl group and an amino group to the other, thereby extending the linker by about 2 Å.

Insertion or deletion of methylene, amino and/or carboxylic acid groups may be desirable in order to optimize the spacing provided by a particular linker.

Anthracene is highly hydrophobic. Thus, more soluble and more easily degraded derivatives, such as anthraquinone, may be more appropriate. In addition to derivatizing the anthracene nucleus, placement of one or more heteroatoms in the ring system may be advantageous to improve solubility and reduce toxicity. Attaching one or more easily ionized groups (e.g. $-SO_3^-$ or $-CH_2-NH_3^+$) to the aromatic nucleus may be useful. Neutral solubilizing groups such as $-CH_2OH$ may also be useful. EpiNEs that bind to HNE with high affinity have net positive charge, favoring amine groups as solubilizing groups.

Other frameworks that may be appropriate include: tetracycline (particularly 2,10 derivatives) (The Pharmacological Basis of Therapeutics, Eighth Edition, Editors Gilman, Rall, Nies, and Taylor, Permagon Press, 1990, ISBN 0-08-040296-8 (hereinafter GOOD-8), p.1117), phenothiazines (p.396, GOOD-8), marprotiline (p.407, GOOD-8), carbamazepine (p.447, GOOD-8), and apomorphine (p.473, GOOD-8). In each case, diametrically opposed positions on the rigid framework are utilized. In designing a linker, groups attached to the framework that engender unwanted or unneeded pharmacological properties are removed.

A further example of a suitable bridging structure would be -Pro-Pro-Pro-.

Figure 12 shows the form of Class III inhibitors. These molecules, like Class I inhibitors, are linear. They lack P5...P1 except that a boronic acid group is positioned to fit into th "active site" of HNE. The boron atom is electrophilic, like the carbonyl carbon of the "normal" P_1 mino acid, and would act similarly. The boronic acid group may be free ($D_1 = D_2 = -OH$) or the boronic acid group may be esterified. Boronic acid esters are readily hydrolyzed in serum. Note that the $-B(OH)-CH_2-$ replaces the scissile bond, too.

The choices for R3, R4, R5 and R6 are the same as for the Class I inhibitors.

For in vivo use, the inhibitor may be administered by, e.g.,

absorption, ingestion, inhalation, or injection, and, if by injection, intravenously, intramuscularly, subcutaneously, etc. The drug may be formulated into any suitable dosage form, such as a tablet, capsule, ointment, syrup, elixir, inhalant, or controlled release implant. For dosage forms, see the current edition of Remington's Pharmaceutical Sciences. The proper dosage may be determined by beginning with a very low dose, and increasing the dosage until the desired inhibitory effect is observed, or by any other means known in the pharmaceutical effect. The inhibitor may be administered to mammalian subjects suffering from excessive neutrophil elastase activity, especially human subjects.

Peptide and protein inhibitors according to the present invention may be prepared by any art-recognized technique, including expression of a corresponding gene or a gene encoding a cleavable fusion protein) in a host cell (see Sambrook, et al.), semisynthesis based on a related protein (see work of Tschesche), or direct organic synthesis. Peptide linkages may be generated using Fmoc, tBoc, or other peptide synthetic chemistry; see SOLID PHASE PEPTIDE SYNTHESIS: A Practical Approach (E. Atherton and R. C. Sheppard, IRL Press at Oxford University, Oxford, England, 1989, ISBN 0-19-963067-4), THE PRACTICE OF PEPTIDE SYNTHESIS (M. Bodanszky and A. Bodanszky, Springer-Verlag, New York, 1984, ISBN 0-387-13471-9), or PRINCIPLES OF PEPTIDE SYNTHESIS (M. Bodanszky, Springer-Verlag, New York, 1984).

These small proteins and derivatized peptides which bind elastase or cathepsin G, regardless of their inhibitory activity, may be useful in purifying the enzymes. However, the preferred compounds are those which are also useful as inhibitors of human neutrophil elastase or cathepsin G, in vitro and in vivo.

Reference Example

Affinity Measurements

The affinity of a protein for another molecule can be measured in many ways. Scatchard (*Ann NY Acad Sci* (1949) 51:660-669)

described a classical method of measuring and analysing binding which has been applied to the binding of proteins. This method requires relatively pure protein and the ability to distinguish bound protein from unbound.

A second method appropriate for measuring the affinity of inhibitors for enzymes is to measure the ability of the inhibitor to slow the action of the enzyme. This method requires, depending on the speed at which the enzyme cleaves substrate and the availability of chromogenic or fluorogenic substrates, tens of micrograms to milligrams of relatively pure inhibitor.

A third method of determing the affinity of a protein for a second material is to have the protein displayed on a genetic package, such as M13, an measure the ability of the protein to adhere to the immobilized "second material". This method is highly sensitive because the genetic packages can be amplified. This approach is not entirely new. Makela, O, H Sarvas, and I Seppala ("Immunological Methods Based on Antigen-Coupled Bacteriophages.", *J Immunol Methods* (1980), 37:213-223) discuss methods of using haptans chemically conjugated to bacteriophage to measure the concentration of antibodies having affinity for the haptans. The present invention uses a novel approach, in that the binding protein is genetically encoded by the phage. Furthermore, we obtain at least semiquantitative values for the binding constants by use of a pH step gradiant. Inhibitors of known affinity for the immobilized protease are used to establish standard profiles against which other phage-displayed inhibitors are judged. Table 203 shows the profile of BPTI-phage and of BPTI(K15L)-phage when these phage are eluted from immobilized hNE. The profiles may vary from one batch of immobilized protease to the next and with the age of the immobilized preparation. Nevertheless, the relative shapes of profiles allow us to identify superior inhibitors.

Ascenzi *et al.* (ASCE90) studied the thermodynamics of binding of BPTI to human and bovine clotting factor X_a. They found that

K_A dropped more than 30-fold as the pH was lowered from 9 to 5. That K_A changes with pH is likely to be general to the binding of serine proteases to KuDoms (and other inhibitors) because of the histidine found in the active site. The pH at which these changes occur is characteristic for the particular protease and inhibitor. It can be seen that protonating the active-site histidine when an inhibitor is bound involves burying a charge, usually energetically unfavorable. The reciprocal effect is that an inhibitor that binds very tightly effectively lowers the pK_a of the imidazole for protonation.

Throughout the present specification, shaken incubations used Labquake shakers.

Preparation of Immobilized Human Neutrophil Elastase

One ml of Reacti-Gel 6 x CDI activated agarose (Pierce Chemical Co.) in acetone (200 µl packed beads) was introduced into an empty Select-D spin column (5Prime-3Prime). The acetone was drained out and the beads were washed twice rapidly with 1.0 ml of ice cold water and 1.0 ml of ice cold 100 mM boric acid, pH 8.5, 0.9% NaCl. Two hundred µl of 2.0 mg/ml human neutrophil elastase (hNE) (CalBiochem, San Diego, CA) in borate buffer were added to the beads. The column was sealed and mixed end over end on a Labquake Shaker at 4°C for 36 hours. The hNE solution was drained off and the beads were washed with ice cold 2.0 M Tris, pH 8.0 over a 2 hour period at 4°C to block remaining reactive groups. A 50% slurry of the beads in TBS/BSA was prepared. To this was added an equal volume of sterile 100% glycerol and the beads were stored as a 25% slurry at -20°C. Prior to use, the beads were washed 3 times with TBS/BSA and a 50% slurry in TBS/BSA was prepared.

EXAMPLE I

CHARACTERIZATION AND FRACTIONATION OF CLONALLY PURE POPULATIONS OF PHAGE, EACH DISPLAYING A SINGLE CHIMERIC APROTININ HOMOLOGUE/M13 GENE III PROTEIN:

This Example demonstrates that chimeric phage proteins displaying a target-binding domain can be eluted from immobilized target by decreasing pH, and the pH at which the protein is eluted indicates the binding affinity of the domain for the target.

Standard Procedures:

Unless otherwise noted, all manipulations were carried out at room temperature. Unless otherwise noted, all cells are XL1-Blue™ (Stratagene, La Jolla, CA).

1) Demonstration of the Binding of BPTI-III MK Phage to Active Trypsin Beads

We demonstrated that BPTI-III display phage bind immobilized active trypsin. Demonstration of the binding of display phage to immobilized active protease and subsequent recovery of infectious phage with a characteristic pH elution profile facilitates evaluation of particular mutants because one need not produce and purify tens of micrograms of each mutant protein.

Phage MK is derived from M13 by inserting a *kan*^{R} gene into the intergenic region. BPTI-III MK phage are derived from MK by inserting into gene *III*, between the codons specifiying the signal sequence and those specifying the mature protein, DNA encodine BPTI. Phage MA is derived from M13 by inserting an *amp*^{R} gene into the intergenic region; phage BPTI-III MA is derived from phage MA by inserting *bpti* into *III* between the signal peptide and mature III encoding regions. BPTI-III MK and BPTI-III MA display BPTI fused to the amino terminus of the gene III protein, about five copies per virion.

Fifty μl of BPTI-III MK phage ($3.7 \cdot 10^{11}$ pfu/ml) in either 50 mM Tris, pH 7.5, 150 mM NaCl, 1.0 mg/ml BSA (TBS/BSA) buffer or 50 mM sodium citrate, pH 6.5, 150 mM NaCl, 1.0 mg/ml BSA (CBS/BSA) buffer were added to 10 μl of a 25% slurry of immobilized trypsin (Pierce Chemical Co., Rockford, IL) also in TBS/BSA or CBS/BSA. As a control, 50 μl MK phage ($9.3 \cdot 10^{12}$ pfu/ml) were added to 10 μl of a 25% slurry of immobilized trypsin in either TBS/BSA or CBS/BSA buffer. The infectivity of BPTI-III MK phage is 25-fold

lower than that of MK phage; thus the conditions chosen above ensure that an approximately equivalent number of phage particles are added to the trypsin beads. After 3 hours of mixing on a Labquake shaker (Labindustries Inc., Berkeley, CA) 0.5 ml of either TBS/BSA or CBS/BSA was added where appropriate to the samples. Beads were washed for 5 min and recovered by centrifugation for 30 sec. The supernatant was removed and 0.5 ml of TBS/0.1% Tween-20 was added. The beads were mixed for 5 minutes on the shaker and recovered by centrifugation. The supernatant was removed and the beads were washed an additional five times with TBS/0.1% Tween-20 as described above. Finally, the beads were resuspended in 0.5 ml of elution buffer (0.1 M HCl containing 1.0 mg/ml BSA adjusted to pH 2.2 with glycine), mixed for 5 minutes and recovered by centrifugation. The supernatant fraction was removed and neutralized by the addition of 130 µl of 1 M Tris, pH 8.0. Aliquots of the neutralized eluate were diluted in LB broth and titered for plaque-forming units.

Table 201 illustrates that a significant percentage of the input BPTI-III MK phage bound to immobilized trypsin and was recovered by washing with elution buffer. The amount of fusion phage which bound to the beads was greater in TBS buffer (pH 7.5) than in CBS buffer (pH 6.5). This is consistent with the observation that the affinity of BPTI for trypsin is greater at pH 7.5 than at pH 6.5 (VINC72, VINC74). A much lower percentage of the MK control phage (no displayed BPTI) bound to immobilized trypsin and this binding was independent of pH. At pH 6.5, 1675 times more of the BPTI-III MK phage than of the MK phage bound to trypsin beads while at pH 7.5, a 2103-fold difference was observed. Hence fusion phage displaying BPTI adhere to active trypsin beads and can be recovered as infectious phage.

Generation of P1 Mutants of BPTI

To demonstrate the specificity of interaction of BPTI-III fusion phage with immobilized serine proteases, single amino acid substitutions were introduced at the P1 position (residue 15 of BPTI) of the BPTI-III fusion protein. The K15L alteration is

desired because BPTI(K15L) is a moderately good inhibitor of human neutrophil elastase (HNE)(K_d = $2.9 \cdot 10^{-9}$ M) (BECK88b) and a poor inhibitor of trypsin. Fusion phage displaying BPTI(K15L) bind to immobilized HNE but not to immobilized trypsin. BPTI-III MK fusion phage display the opposite phenotype (bind to trypsin, fail to bind to HNE). These observations illustrate the binding specificity of BPTI-III fusion phage for immobilized serine proteases.

Characterization of the Affinity of BPTI-III MK and BPTI(K15L)-III MA Phage for Immobilized Trypsin and Human Neutrophil Elastase

Thirty μl of BPTI-III MK phage in TBS/BSA ($1.7 \cdot 10^{11}$ pfu/ml) was added to 5 μl of a 50% slurry of either immobilized human neutrophil elastase or immobilized trypsin (Pierce Chemical Co.) also in TBS/BSA. Similarly, 30 μl of BPTI(K15L)-III MA phage in TBS/BSA ($3.2 \cdot 10^{10}$ pfu/ml) was added to either immobilized HNE or trypsin. Samples were mixed on a Labquake shaker for 3 hours. The beads were washed with 0.5 ml of TBS/BSA for 5 minutes and recovered by centrifugation. The supernatant was removed and the beads were washed 5 times with 0.5 ml of TBS/0.1% Tween-20. Finally, the beads were resuspended in 0.5 ml of elution buffer (0.1 M HCl containing 1.0 mg/ml BSA adjusted to pH 2.2 with glycine), mixed for 5 minutes and recovered by centrifugation. The supernatant fraction was removed, neutralized with 130 μl of 1 M Tris, pH 8.0, diluted in LB broth, and titered for plaque-forming units.

Effect of pH on the Dissociation of Bound BPTI-III MK and BPTI(K15L)-III MA Phage from Immobilized Neutrophil Elastase

The affinity of a given fusion phage for an immobilized serine protease can be characterized on the basis of the amount of bound fusion phage which elutes from the beads by washing with a step gradient that goes, for example, from about pH 7.0 to about pH 2.2 in steps of 1 or 0.5 pH units. Since the affinity of the above described BPTI variants for HNE is not high (K_d > $1 \cdot 10^{-9}$ M), we

anticipated that fusion phage displaying these variants might dissociate from HNE beads at a pH above 2.2. Furthermore fusion phage might dissociate from HNE beads at a specific pH characteristic of the particular BPTI variant displayed. Low pH buffers providing stringent wash conditions might be required to dissociate fusion phage displaying a BPTI variant with a high affinity for HNE whereas neutral pH conditions might be sufficient to dislodge a fusion phage displaying a BPTI variant with a weak affinity for HNE.

Thirty μl of BPTI(K15L)-III MA phage ($1.7 \cdot 10^{10}$ pfu/ml in TBS/BSA) were added to 5 μl of a 50% slurry of HNE beads also in TBS/BSA. Similarly, 30 μl of BPTI-III MA phage ($8.6 \cdot 10^{10}$ pfu/ml in TBS/BSA) were added to 5 μl of HNE beads. Thus, an approximately equivalent number of phage particles were added to the beads. Samples were incubated for 3 hours with shaking. The beads were washed with 0.5 ml of TBS/BSA for 5 min with shaking, recovered by centrifugation, and the supernatant removed. The beads were washed with 0.5 ml of TBS/0.1% Tween-20 for 5 minutes and recovered by centrifugation. Four additional washes with TBS/0.1% Tween-20 were performed. The beads were washed with 0.5 ml of 100 mM sodium citrate, pH 7.0 containing 1.0 mg/ml BSA. The beads were recovered by centrifugation and the supernatant was removed. The HNE beads were washed sequentially with a series of 100 mM sodium citrate, 1.0 mg/ml BSA buffers of pH 6.0, 5.0, 4.0 and 3.0 and finally with the 2.2 elution buffer. The pH washes were neutralized by the addition of 1 M Tris, pH 8.0, diluted in LB broth and titered for plaque-forming units.

Table 203 illustrates that a low percentage of the input BPTI-III MK fusion phage adhered to the HNE beads and was recovered in the pH 7.0 and 6.0 washes predominantly. A significantly higher percentage of the BPTI(K15L)-III MA phage bound to the HNE beads and was recovered predominantly in the pH 5.0 and 4.0 washes. Hence lower pH conditions (*i.e.* more stringent) are required to dissociate BPTI(K15L)-III MA than BPTI-MK phage from immobilized

HNE. The affinity of BPTI(K15L) is over 1000 times greater than that of BPTI for HNE (using reported K_d values (BECK88b)). Hence this suggests that lower pH conditions are required to dissociate fusion phage displaying a BPTI variant with a higher affinity for HNE.

Effect of Mutation of Residues 39-42 of BPTI(K15L) on its Affinity for Immobilized HNE

Thirty μl of BPTI(K15L,MGNG)-III MA phage ($9.2 \cdot 10^9$ pfu/ml in TBS/BSA) were added to 5 μl of a 50% slurry of immobilized HNE also in TBS/BSA. Similarly 30 μl of BPTI(K15L)-III MA phage ($1.2 \cdot 10^{10}$ pfu/ml in TBS/BSA) were added to immobilized HNE. The samples were incubated for 3 hours with shaking. Beads were washed for 5 min with 0.5 ml TBS/BSA and spun down. The beads were washed 5 times with 0.5 ml TBS/0.1% Tween-20. Finally, the beads were washed sequentially with a series of 100 mM sodium citrate buffers of pH 7.0, 6.0, 5.5, 5.0, 4.75, 4.5, 4.25, 4.0 and 3.5. pH washes were neutralized, diluted in LB broth and titered for plaque-forming units.

Table 204 illustrates that almost twice as much of BPTI(K15L,MGNG)-III MA as BPTI(K15L)-III MA phage bound to HNE beads. In both cases the pH 4.75 fraction contained the largest proportion of recovered phage confirming that replacement of residues 39-42 of wild type BPTI with M_{39}GNG from BI-8e enhances the binding of the BPTI(K15L) variant to HNE.

Construction of BPTI(K15V,R17L)-III MA

BPTI(K15V,R17L) demonstrates the highest affinity for HNE of any BPTI variant yet described ($K_d = 6 \cdot 10^{-11}$ M) (AUER89). To test the elution system, a phage displaying this BPTI(K15V,R17L) was generated and used as a reference phage to characterize the affinity for immobilized HNE of fusion phage displaying a BPTI

variant with a known affinity for free HNE.

Affinity of BPTI(K15V,R17L)-III MA Phage for Immobilized HNE

Forty μl of BPTI(K15,R17L)-III MA phage ($9.8 \cdot 10^{10}$ pfu/ml) in TBS/BSA were added to 10 μl of a 50% slurry of immobilized HNE also in TBS/BSA. Similarly, 40 μl of BPTI(K15L,MGNG)-III MA phage ($5.13 \cdot 10^{9}$ pfu/ml) in TBS/BSA were added to immobilized HNE. The samples were shaken for 1.5 hours. Beads were washed once for 5 min with 0.5 ml of TBS/BSA and then 5 times with 0.5 ml of TBS/1.0% Tween-20. The beads were then washed sequentially with a series of 50 mM sodium citrate buffers containing 150 mM NaCl, 1.0 mg/ml BSA of pH 7.0, 6.0, 5.0, 4.5, 4.0, 3.75, 3.5 and 3.0. For BPTI(K15L,MGNG)-III MA, the pH 3.75 and 3.0 washes were omitted. Two washes were performed at each pH and the supernatants pooled, neutralized with 1 M Tris pH 8.0, diluted in LB broth, and titered for plaque-forming units.

Table 206 illustrates that the pH 4.5 and 4.0 fractions contained the largest proportion of the recovered BPTI(K15V,R17L)-III MA phage. BPTI(K15L,MGNG)-III MA phage, like BPTI(K15L)-III MA phage, were recovered predominantly in the pH 5.0 and 4.5 fractions, as above. The affinity of BPTI(K15V,R17L) is 48 times greater than that of BPTI(K15L) for HNE (using K_d values, AUER89 for BPTI(K15V,R17L) and BECK88b for BPTI(K15L)). That the pH elution profile for BPTI(K15V,R17L)-III MA phage exhibits a peak at pH 4.0 while the profile for BPTI(K15L)-III MA phage displays a peak at pH 4.5 supports the contention that lower pH conditions are required to dissociate, from immobilized HNE, fusion phage displaying a BPTI variant with a higher affinity for free HNE.

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EXAMPLE II

BPTI Derivatives having high affinity for hNE

We caused BPTI mutants to appear on the surface of M13-derived display phage as amino-terminal fusions to the gene *III* protein (gIIIp); M13 has about five copies of gIIIp per virion. Our phage library theoretically included the 1728 BPTI mutants with PHE,

LEU, ILE, VAL or MET at positions 15 and 17, GLY or ALA at position 16, PHE, SER, THR or ILE at position 18 and SER, PRO, THR, LYS or GLN at position 19, as a result of expression of a BPTI gene (coding for the aforementioned MGNG mutation) subjected to controlled random mutagenesis, and screened for hNE-binding activity by incubating phage bearing the mutants with immobilized hNE, and eluting the phage with progressively more acidic buffers. Twenty mutants (see clonal identifiers in Tables 207-208) were selected for sequencing, and exhibited eight unique sequences. Tables 207 and 208 show the sequences of nine (the eight, plus another identified in a pilot study) BPTI derivatives having high affinity for hNE. EpiNE1, EpiNE3, EpiNE5, EpiNE6 and EpiNE7 eluted at pH 3.5; EpiNE2, EpiNE4, and EpiNE8 at pH 3.5-4.

That pH conditions less than 4.0 are required to elute EpiNE1, EpiNE3, and EpiNE7-bearing phage from immobilized HNE suggests that they display BPTI variants having a higher affinity for HNE than BPTI(K15V,R17L).

EpiNE1, EpiNE3 and EpiNE7 were expressed as soluble proteins and analyzed for HNE inhibition activity by the fluorometric assay of Castillo et al. (CAST79); the data were analyzed by the method of Green and Work (GREE53). EpiNE1, EpiNE3, and EpiNE7 have been produced as free proteins, both in *E. coli* and in yeast. The ability of these proteins to inhibit hNE was measured by following the cleavage of a fluorogenic substrate. The K_i for these compounds is 1 pM, 3 pM, and 3 pM. Phage that display EpiNE1 are used to establish a reference pH-elution profile to allow quick characterization of other KuDom inhibitors displayed on phage. All of the listed EpiNEs have lower K_ds than BPRI(K15V,R17L)(60 pM).

An examination of the sequences of the EpiNE clones is illuminating. A strong preference for either VAL or ILE at the P1 position (residue 15) is indicated with VAL being favored over ILE by 14 to 6. No examples of LEU, PHE, or MET at the P1 position were observed although the screened library theoretically should

have included mutants with these amino acids at P1. This is consistent with the observation that BPTI variants with single amino acid substitutions of LEU, PHE, or MET for LYS_{15} exhibit a significantly lower affinity for HNE than their counterparts containing either VAL or ILE (BECK88b).

PHE is strongly favored at position 17, appearing in 12 of 20 clones. MET is the second most prominent residue at this position but it only appears when VAL is present at position 15. At position 18 PHE was observed in all 20 clones sequenced even though the library should have included other residues at this position. This result is quite surprising and could not be predicted from previous mutational analysis of BPTI, model building, or on any theoretical grounds. We infer that the presence of PHE at position 18 significantly enhances the ability each of the EpiNEs to bind to HNE. Finally at position 19, PRO appears in 10 of 20 codons while SER, the second most prominent residue, appears at 6 of 20 codons. Of the residues targeted for mutagenesis in the present study, residue 19 is the nearest to the edge of the interaction surface of an inhibitor with HNE. Nevertheless, a preponderance of PRO is observed and may indicate that PRO at 19, like PHE at 18, enhances the binding of these proteins to HNE. Interestingly, EpiNE5 appears only once and differs from EpiNE1 only at position 19; similarly, EpiNE6 differs from EpiNE3 only at position 19. These alterations may have only a minor effect on the ability of these proteins to interact with HNE. This is supported by the fact that the pH elution profiles for EpiNE5 and EpiNE6 are very similar to those of EpiNE1 and EpiNE3 respectively.

Only EpiNE2 and EpiNE8 exhibit pH profiles which differ from those of the other selected clones. Both clones contain LYS at position 19 which may restrict the interaction of BPTI with HNE. However, we can not exclude the possibility that other alterations within EpiNE2 and EpiNE8 (R15L and Y21S respectively) influence their affinity for HNE.

Position 18 has not previously been identified as a key position in determining specificity or affinity of aprotinin homologues or derivatives for particular serine proteases. None have reported or suggested that phenylalanine at position 18 will confer specificity and high affinity for HNE.

EXAMPLE III

BPTI Derivatives having high affinity for hCG

The same library of BPTI mutant-bearing phage was also screened for Cathepsin G binding activity. Figure 7 shows the binding and pH profiles for the individual Cat G binding clones (designated EpiC variants). All clones exhibited minor peaks, superimposed upon a gradual fall in bound phage, at pH elutions of 5 (clones 1, 8, 10 and 11) or pH 4.5 (clone 7). Table 209 reports clones that show binding to Cat G beads.

A comparison of the pH profiles elicited for the EpiC variants with Cat G and the EpiNE variants for hNE indicates that the EpiNE variants have a high affinity for hNE while the EpiC variants have a moderate affinity for Cat G.

The P1 residue in the EpiC mutants is predominantly MET, with one example of PHE, while in BPTI it is LYS and in the EpiNE variants it is either VAL or LEU. In the EpiC mutants residue 16 is predominantly ALA with one example of GLY and residue 17 is PHE, ILE or LEU. Interestingly residues 16 and 17 appear to pair off by complementary size, at least in this small sample. The small GLY residue pairs with the bulky PHE while the relatively larger ALA residue pairs with the less bulky LEU and ILE. The majority of the available residues in the MYMUT library for positions 18 and 19 are represented in the EpiC variants.

EXAMPLE IV

ITI:D1 Derivatives having high affinity for hNE

Construction of the display vector.

We use the nucleic-acid numbering of the *ITI-light-chain* gene found in TRAB86 and the amino-acid numbering shown for UTI in Fig. 1 of GEBH86. We manipulated DNA according to standard methods as

described in SAMB89 and AUSU87.

The protein sequence of human ITI-D1 consists of 56 amino acid residues extending from LYS_{22} to ARG_{77} of the complete ITI light chain sequence. This sequence is encoded by the 168 bases between positions 750 and 917 in the cDNA sequence presented in TRAB86. DNA encoding this amino-acid sequence was introduced into M13 gene *iii* by standard means. Phage isolates containing the ITI-D1-III fusion gene are called MA-ITI and carry an amp^R gene. Expression of the ITI-D1::III fusion protein and its display on the phage surface were demonstrated by Western analysis and phage-titer neutralization experiments with rabbit anti(hITI) serum.

Fractionation of MA-ITI phage bound to agarose-immobilized protease beads.

To test if phage displaying the ITI-D1-III fusion protein interact strongly with the proteases human neutrophil elastase (hNE) or cathepsin-G, aliquots of display phage were incubat d with agarose-immobilized hNE or cathepsin-G beads (hNE beads or Cat-G beads, respectively). The beads were washed and bound phage eluted by pH fractionation. The procession in lowering pH was: pH 7.0, 6.0, 5.5, 5.0, 4.5, 4.0, 3.5, 3.0, 2.5, and 2.0. Following elution and neutralization, the various input, wash, and pH elution fractions were titered.

The results of several fractionations are summarized in Table 212 (EpiNE-7 or MA-ITI phage bound to hNE beads) and Table 213 (EpiC-10 or MA-ITI phage bound to Cat-G beads). For the two types of beads (hNE or Cat-G), the pH elution profiles obtained using the control display phage (EpiNE-7 or EpiC-10, respectively) were similar to those seen previously. About 0.3% of the EpiNE-7 display phage applied to the hNE beads were eluted during the fractionation procedure and the elution profile had a maximum for elution at about pH 4.0. A smaller fraction, 0.02%, of the EpiC-10 phage applied to the Cat-G beads were eluted and the elution profile displayed a maximum near pH 5.5.

The MA-ITI phage show no evidence of great affinity for either

hNE or cathepsin-G immobilized on agarose beads. The pH elution profiles for MA-ITI phage bound to hNE or Cat-G beads show essentially monotonic decreases in phage recovered with decreasing pH . Further, the total fractions of the phage applied to the beads that were recovered during the fractionation procedures were quite low: 0.002% from hNE beads and 0.003% from Cat-G beads.

Published values of K_i for inhibition neutrophil elastase by the intact, large (M_r=240,000) ITI protein range between 60 and 150 nM and values between 20 and 6000 nM have been reported for the inhibition of Cathepsin G by ITI (SWAI88, ODOM90). Our own measurements of pH fraction of display phage bound to hNE beads show that phage displaying proteins with low affinity (>μM) for hNE are not bound by the beads while phage displaying proteins with greater affinity (nM) bind to the beads and are eluted at about pH 5. If the first KuDom of the ITI light chain is entirely responsible for the inhibitory activity of ITI against hNE, and if this domain is correctly displayed on the MA-ITI phage, then it appears that the minimum affinity of an inhibitor for hNE that allows binding and fractionation of display phage on hNE beads is 50 to 100 nM.

Alteration of the P1 region of ITI-D1.

If ITI-D1 and EpiNE-7 assume the same configuration in solution as BPTI, then these two polypeptides have identical amino acid sequences in both the primary and secondary binding loops with the exception of four residues about the P1 position and at positions 11 and 34. For ITI-D1 the sequence for positions 15 to 20 is (position 15 in ITI-D1 corresponds to position 36 in the UTI sequence of GEBH86):

	BPTI position numbers									hNE
Domain	11	15	16	17	18	19	20	31	34	Affinity
EpiNE7	T	V	A	M	F	P	R	Q	V	very high
ITI-D1	A	M	G	M	T	S	R	E	Q	modest
	32	36	37	38	39	40	41	52	55 ←	ITI positions

These two proteins differ greatly in their affinities for hNE. To improve the affinity of ITI-D1 for hNE, the EpiNE-7 sequence was

incorporated by cassette mutagenesis into the ITI-D1 sequence at positions 15 through 20. Phage containing the ITI-D1-III fusion gene with the EpiNE-7 changes around the P1 position are called MA-ITI-E7.

Fractionation of MA-ITI-E7 phage.

To test if the changes at positions 15, 16, 18, and 19 of the ITI-D1-III fusion protein influence binding of display phage to hNE beads, abbreviated pH elution profiles were measured. Aliquots of EpiNE-7, MA-ITI, and MA-ITI-E7 display phage were incubated with hNE beads for three hours at room temperature. Th beads were washed and phage were eluted as described above, except that only three pH elutions were performed: pH 7.0, 3.5, and 2.0. The results of these elutions are shown in Table 214.

Binding and elution of the EpiNE-7 and MA-ITI display phage were found to be as described. The total fraction of input phages was high (0.4%) for EpiNE-7 phage and low (0.001%) for MA-ITI phage. Further, the EpiNE-7 phage showed maximum phage elution in the pH 3.5 fraction while the MA-ITI phage showed only a monotonic decrease in phage yields with decreasing pH , as seen above.

MA-ITI-E7 phage show increased levels of binding to hNE beads relative to MA-ITI phage. The total fraction of the input phage eluted from the beads is 10-fold greater for both MA-ITI-E7 phage strains than for MA-ITI phage (although still 40-fold lower that EpiNE-7 phage). Further, the pH elution profiles of the MA-ITI-E7 phage strains show maximum elutions in the pH 3.5 fractions, similar to EpiNE-7 phage.

To further define the binding properties of MA-ITI-E7 phage, the extended pH fractionation procedure described previously was performed using phage bound to hNE beads, as shown in Table 215. The pH elution profile of EpiNE-7 display phage is as previously described. In this more resolved pH elution profile, MA-ITI-E7 phage show a broad elution maximum centered around pH 5. Again, the total fraction of MA-ITI-E7 phage obtained on pH elution from hNE beads was about 40-fold less than that obtained using EpiNE-7 display phage.

The pH elution behavior of MA-ITI-E7 phage bound to hNE beads is qualitatively similar to that seen using BPTI[K15L]-III-MA phage. BPTI with the K15L mutation has an affinity for hNE of $\sim 3. \cdot 10^{-9}$ M. Assuming all else remains the same, the pH elution profile for MA-ITI-E7 suggests that the affinity of the free ITI-D1-E7 domain for hNE is in the nM range. Thus, the substitution of the EpiNE-7 sequence in place of the ITI-D1 sequence around the P1 region has produced an apparent 20- to 50-fold increased affinity for hNE (assuming K_i = 60 to 150 nM for ITI-D1).

If EpiNE-7 and ITI-D1-E7 have the same solution structure, these proteins present the identical amino acid sequences to hNE over the interaction surface. Despite this similarity, EpiNE-7 exhibits a roughly 1000-fold greater affinity for hNE than does ITI-D1-E7. This observation highlights the importance of non-contacting secondary residues in modulating interaction strengths.

ITI light chain is glycosylated at SER10 and ASN45 (GEBH86). Removal of the glycosaminoglycan chains has been shown to decrease the affinity of the inhibitor for hNE about 5-fold (SELL87). Another potentially important difference between EpiNE-7 and ITI-D1-E7 is that of net charge. BPTI has charge +6 while EpiNE7 has charge +1 and ITI-D1 has charge -1. Furthermore, the change in charge between these two molecules arises from differences in the central portions of the molecules which neighbors the binding surface. Position 26 is LYS in EpiNE-7 and is THR in ITI-D1-E7, while at position 31 the residues are GLN and GLU, respectively. These sequence changes not only alter the net molecular charge but also place negative charge close to the interaction surface in ITI-D1-E7. It may be that the occurrence of a negative charge at position 31 (not found in any other hNE inhibitors here described) destabilized the inhibitor-protease interaction.

Preparation of BITI-E7 Phage

We replaced K_1EDS of ITI-D1 with R_1PDF from EpiNE7 to make phage MA-BITI-E7. Phe_4 of BPTI is part of the hydrophobic core of

the protein; replacement with serine may alter the stability or dynamic character of ITI-E7 unfavorably. ITI-E7 has a negatively charged Glu at position 2 while EpiNe7 has Pro.

We made the same changes at the putative amino terminus of the ITI-III fusion protein displayed by the phage MA-ITI. These phage are called MA-BITI.

We compared the properties of the ITI-III fusion proteins displayed by phage MA-ITI and MA-BITI using Western analysis. We found no significant differences in apparent size or relative abundance of the fusion proteins produced by either display phage strain. Thus, there are no large differences in the processed forms of either fusion protein displayed on the phage. By extension, there are also no large differences in the processed forms of the gene III fusion proteins displayed by MA-ITI-E7 and MA-EpiNE7. Large changes in protein conformation due to greatly altered processing are therefore not likely to be responsible for the great differences in binding to hNE-beads shown by MA-ITI-E7 and MA-EpiNE7 display phage.

We characterized the binding properties to hNE-beads of MA-BITI and MA-BITI-E7 display phage using the extended pH fractionation procedure described previously, see Table 216. The pH elution profile of MA-EpiNE7 display phage bound to hNE-beads is similar to that previously described. The pH elution profiles for MA-BITI and MA-BITI-E7 show significant differences from the profiles exhibited by MA-ITI and MA-ITI-E7 (*cf.* Tables 212 and 215). In both cases, the alterations at the putative amino terminus of the displayed fusion protein produce a several-fold increase in the fraction of the input display phage eluted from the hNE-beads.

The binding capacity of hNE-beads for display phage varies among preparations of beads and with age for each individual preparation of beads. Thus, one should compare the relative shapes of profiles obtained on beads of substantially the same age and from the same batch. For example, the fraction of MA-EpiNE7 display phage recovered from hNE-beads varies two-fold among the

experiments shown in Tables 212, 215, and 216, and from results given elsewhere in the present specification. However, the shapes of the pH elution profiles are quite similar. It is possible to correct approximately for variations in binding capacity of hNE-beads by normalizing display phage yields to the total yield of MA-EpiNE7 phage recovered from the beads in a concurrent elution. When the data shown in Tables 212, 215, and 216 are so normalized, the recoveries of display phage, relative to recovered MA-EpiNE7, are:

display phage strain	normalized fraction of input
MA-ITI	0.0067
MA-BITI	0.027
MA-ITI-E7	0.027
MA-BITI-E7	0.13

Thus, the alterations in the amino terminal sequence of the displayed fusion protein produce a three- to five-fold increase in the fraction of phage eluted from hNE-beads. While the MA-ITI-E7 elute with a broad pH maximum centered around pH 5.0, the pH elution profile for MA-BITI-E7 phage has a pH maximum at around pH 4.75 to pH 4.5.

The pH elution maximum of the MA-BITI-E7 display phage is located between the maxima exhibited by the BPTI(K15L) and BPTI(K15V, R17L) display phage (pH 4.75 and pH 4.5 to pH 4.0, respectively) described previously (Example III). From the pH maximum exhibited by the display phage we estimate that the BITI-E7 protein free in solution has an affinity for hNE in the 10^{-10} M range. This would represent an approximately ten-fold increase in affinity for hNE over that estimated above for ITI-E7.

As described above, Western analysis of phage proteins show that there are no large changes in gene III fusion proteins upon alteration of the amino terminal sequence. Thus, it is unlikely that the changes in affinity of display phage for hNE-beads can be attributed to large-scale alterations in protein folding resulting

from altered ("correct") processing of the fusion protein in the amino terminal mutants. The improvements in binding may in part be due to: 1) the decrease in the net negative charge (-1 to 0) on the protein arising from the GLU to PRO change at position 2, or 2) increased protein stability resulting from the SER to PHE substitution at residue 4 in the hydrophobic core of the protein, or 3) the combined effects of both substitutions.

Production and properties of MA-BITI-E7-1222 and MA-BITI-E7-141

Within the presumed KuDom:hNE interface, BITI-E7 and EpiNE7 differ at only two positions: 11 and 34. In EpiNE7 these residues are THR and VAL, respectively. In BITI-E7 they are ALA and GLN. In addition BITI-E7 has GLU at 31 while EpiNE7 has GLN. This negative charge may influence binding although the residue is not directly in the interface. We used oligonucleotide-directed mutagenesis to investigate the effects of substitutions at positions 11, 31 and 34 on the protease:inhibitor interaction.

Phage MA-BITI-E7-1222 is the same as BITI-E7 with the mutation A11T. Phage MA-BITI-E7-141 is the same as BITI-E7 with the mutations E31Q and Q34V.

We determined the binding properties to hNE-beads of MA-BITI-E7-1222 and MA-BITI-E7-141 display phage using the extended pH fractionation protocol, as shown in Tables 217 (for MA-BITI-E7 and MA-BITI-E7-1222) and 218 (for MA-EpiNE7 and MA-BITI-E7-141).

Thus, the substitution of THR for ALA at position 11 in the displayed ITI derivative has no appreciable effect on the binding of display phage to hNE-beads.

In contrast, the changes at positions 31 and 34 profoundly affect the hNE-binding properties of the display phage (Table 218). The elution profile pH maximum of MA-BITI-E7-141 phage is shifted to lower pH relative to the parental MA-BITI-E7 phage. Further, the position of the maximum (between pH 4.5 and pH 4.0) is identical to that exhibited by MA-EpiNE7 phage in this experiment. Finally, the MA-BITI-E7-141 phage show a ten-fold increase, relative to the parental MA-BITI-E7, in the total

fraction of input phage eluted from hNE-beads (0.3% vs 0.03%). Indeed, the total fraction of MA-BITI-E7-141 phage eluted from the hNE-beads is nearly twice that of MA-EpiNE7 phage.

The results discussed above show that binding by MA-BITI-E7-141 display phage to hNE-beads is comparable to that of MA-EpiNE7 phage. Thus, BITI-E7-141 may have K_D < 1 pM. Such an affinity is approximately 100-fold greater than that estimated above for the parent (BITI-E7) and is 10^5 to 10^6 times as great as the affinity for hNE reported for the intact ITI protein.

Mutagenesis of BITI-E7-141

BITI-E7-141 differs from ITI-D1 at nine positions (1, 2, 4, 15, 16, 18, 19, 31, and 34). To obtain the protein having the fewest changes from ITI-D1 while retaining high specific affinity for hNE, we have investigated the effects of reversing the changes at positions 1, 2, 4, 16, 19, 31, and 34. The changes we have introduced into the BITI-E7-141 protein are introduced schematically below:

Displayed Protein	residue 1	2	3	4	....	11	....	15	16	17	18	19	..	26	..	31	..	34
ITI-D1	**K**	**E**	**D**	**S**	....	**A**	....	**M**	**G**	**M**	**T**	**S**	..	**T**	..	**E**	..	**Q**
141	R	P	**D**	F	....	**A**	....	V	A	**M**	F	P	..	**T**	..	Q	..	V
MUT1619	R	P	**D**	F	....	**A**	....	V	**G**	**M**	F	**S**	..	**T**	..	Q	..	V
MUTP1	R	P	**D**	F	....	**A**	....	**I**	**G**	**M**	F	**S**	..	**T**	..	Q	..	V
AMINO1	**K**	**E**	**D**	F	....	**A**	....	V	A	**M**	F	P	..	**T**	..	Q	..	V
AMINO2	**K**	P	**D**	**S**	....	**A**	....	V	A	**M**	F	P	..	**T**	..	Q	..	V
MUTQE	R	P	**D**	F	....	**A**	....	V	A	**M**	F	P	..	**T**	..	**E**	..	V
MUTT26A	R	P	**D**	F	....	**A**	....	V	A	**M**	F	P	..	**A**	..	Q	..	V
MUT200	**K**	P	**D**	F	....	**A**	....	V	**G**	**M**	F	**S**	..	A	..	**E**	..	V

ITI-D1 residues are shown in bold type and residues found in neither ITI-D1 nor in BILTI-E7-141 are shown underlined in bold. MUT1619 restores the ITI-D1 residues at positions 16 and 19. It

is likely that MET at 17 and PHE at 18 are optimal for high affinity hNE binding, but $F_{17}F_{18}$ is also effective. GLY at 16 and SER at 19 occurred frequently in the high affinity hNE-binding BPTI-variants obtained from fractionation of a library of BPTI-variants against hNE (ROBE91). Thus, it seems likely that the ITI-D1 sequence at these positions can be restored while maintaining high specific affinity for hNE. The sequence designated MUT200 is hypothetical, but is very likely to have high affininty for hNE.

The BITI display phage were produced by substituting R_1PDF of EpiNE7 for K_1EDS of ITI phage.

Two changes had been introduced into the sequence for BITI-E7 to produce BITI-E7-141: GLU to GLN at position 31 and GLN to VAL at position 34.

The BITI-E7-141 protein sequence ASN24-GLY25-THR26 matches the general recognition sequence ASN-X-THR/SER for N-linked glycosylation in eukaryotic organisms. In the intact ITI molecule isolated from human serum, the light chain polypeptide is glycosylated at this site (ASN45, ODOM90). It is likely that ASN24 will be glycosylated if the BITI-E7-141 protein is produced via eukaryotic expression. Such glycosylation may render the protein difficult to purify to homogeneity and immunogenic when used for long-term treatment. We changed T_{26} to A because alanine is found frequently at this locus in KuDoms.

<u>hNE-binding properties of mutagenized MA-BITI-E7-141 display phage</u>

The binding properties of the individual phage populations to hNE-beads were determined using the abbreviated and extended pH elution protocols described previously. The results of these studies are presented in Table 219.

Table 219 shows pH elution data for the various display phage eluted from hNE-beads. Total pfu applied to the beads are shown in the second column. The fractions of this input pfu recovered in each pH fraction of the abbreviated pH elution protocol (pH 7.0, pH 3.5, and pH 2.0) are listed in the next three columns.

For data obtained using the extended pH elution protocol, the pH 3.5 listing represents the sum of the fractions of input recovered in the pH 6.0, pH 5.5, pH 5.0, pH 4.5, pH 4.0, and pH 3.5 elution samples. Likewise, the pH 2.0 listing is the sum of the fractions of input obtained from the pH 3.0, pH 2.5, and pH 2.0 elution samples. The total fraction of the input pfu obtained throughout the pH elution protocol is recorded in the sixth column of Table 219. The final column of the table lists the total fraction of input pfu recovered normalized to the value obtained for MA-BITI-E7-141 display phage.

Two factors must be considered when making comparisons among the data shown in Table 219. The first is that, due to the kinetic nature of phage release from hNE-beads and the longer time involved in the extended pH elution protocol, the fraction of input pfu recovered in the pH 3.5 fraction will be enriched at the expense of the pH 2.0 fraction in the extended protocol relative to those values obtained in the abbreviated protocol. The magnitude of this effect can be seen by comparing the results obtained when MA-BITI-E7-141 display phage were eluted from hNE-beads using the two protocols. The second factor is that, for the range of input pfu listed in Table 219, the input pfu influences recovery. The greater the input pfu, the greater the total fraction of the input recovered in the elution. This effect is apparent when input pfu differ by more than a factor of about 3 to 4. The effect can lead to an overestimate of affinity of display phage for hNE-beads when data from phage applied at higher titers is compared with that from phage applied at lower titers.

Mindful of these caveats, we interpret Table 219. The effects of the mutations introduced into MA-BITI-E7-141 display phage ("parental") on binding of display phage to hNE-beads can be grouped into three categories: those changes that have little or no effects, those that have moderate (2- to 3-fold) effects, and those that have large (>5-fold) effects.

The MUTT26A and MUTQE changes appear to have little effect on the binding of display phage to hNE-beads. In terms of total pfu

recovered, the display phage containing these alterations bind as well as the parental to hNE-beads. Indeed, the pH elution profiles obtained for the parental and the MUTT26A display phage from the extended pH elution protocol are indistinguishable. The binding of the MUTQE display phage appears to be slightly reduced relative to the parental and, in light of the applied pfu, it is likely that this binding is somewhat overestimated.

The sequence alterations introduced via the MUTP1 and MUT1619 oligonucleotides appear to reduce display phage binding to hNE-beads about 2- to 3-fold. In light of the input titers and the distributions of pfu recovered among the various elution fractions, it is likely that 1) both of these display phage have lower affinities for hNE-beads than do MA-EpiNE7 display phage, and 2) the MUT1619 display phage have a greater affinity for hNE-beads than do the MUTP1 display phage.

The sequence alterations at the amino terminus of BITI-E7-14 appear to reduce binding by the display phage to hNE-beads at least ten fold. The AMINO2 changes are likely to reduce display phage binding to a substantially greater extent than do the AMINO1 changes.

On the basis of the above interpretations of the data listed in Table 219, we can conclude that:

1.) The substitution of ALA for THR at position 26 in ITI-D1 and its derivatives has no effect on the interaction of the inhibitor with hNE. Thus, the possibility of glycosylation at ASN24 of an inhibitor protein produced in eukaryotic cell culture can be avoided with no reduction in affinity for hNE.

2.) The increase in affinity of display phage for hNE-beads produced by the changes GLU to GLN at position 31 and GLN to VAL at 34 results primarily from the VAL substitution at 34.

3.) All three changes introduced at the amino terminal region of ITI-D1 (positions 1,2, and 4) influence display phage binding to hNE-beads to varying extents. The change at position 4 (SER to PHE) appears to have a much greater effect than does the change at

position 2. The change at position 1 may have little or no effect.

4.) The changes in the region around the P1 residue in BITI-E7-141 (position 15) influence display phage binding to hNE. The changes ALA to GLY at 16 and PRO to SER at 19 appear to reduce the affinity of the inhibitor somewhat (perhaps 3-fold). The substitution of ILE for VAL at position 15 further reduces binding.

BITI-E7-141 differs from ITI-D1 at nine positions. On the basis of the discussion above it appears likely that a high affinity hNE-inhibitor based on ITI-D1 could be constructed that would differ from the ITI-D1 sequence at only four or five positions. These differences would be: PHE at position 4, VAL at position 15, PHE at position 18, VAL at position 34, and ALA at position 26. If glycosylation of ASN24 is not a concern THR could be retained at 26.

10. Summary: estimated affinities of isolated ITI-D1 derivatives for hNE

On the basis of display phage binding to and elution from hNE beads, it is possible to estimate affinities for hNE that various derivatives of ITI-D1 may display free in solution. These estimates are summarized below and in Table 220.

Example 5

Figure 11 illustrates a number of initial and intermediate compounds involved in a hypothetical synthetic route of a linker that incorporates R_2 = 2-propyl, X = [-CO-CF2-], and R_3 = $-CH_3$. Compound I is glyceraldehyde in which the hydroxyls are protected by methylthiomethyl (MTM) groups (p.680 in ADVANCED ORGANIC CHEMISTRY, Third Edition, Part B: Reactions and Synthesis, F. A Carey and R. J. Sundberg, Plenum Press, New York, 1990, ISBN 0-306-43456-3 (hereinafter CARE90) and works cited therein). Compound I is reacted with the Grignard reagent formed by 2 propylchloride to yield II. In III, the hydroxyl at

C_3 has been protected by a tetrahydropyranyl ether (THP) group (p.679 in CARE90). The MTM groups are selectively removed under nonacidic conditions in aqueous solution with Ag^+ or Hg^{++} (p.680, CARE90). The hydroxyl at C_2 is selectively oxidized to the ketone with N-bromosuccinimide (p1059 in ADVANCED ORGANIC CHEMISTRY, Reactions, Mechanisms, and Structure Third Edition, Jerry March, John Wiley & Sons, New York, 1985, ISBN 0-471-88841-9 (hereinafter MARC85) and Filler, Chem Rev, 63:21-43 (1963) (FILL63)) to give compound IV.

The C_1 hydroxyl of IV is blocked with MTM, the keto group at C_2 is reduced to the alcohol with $LiAlH_4$ (p.809 MARC85); the C_2 hydroxyl is blocked with a ß-methoxyethoxymethyl group (p.679 CARE90) and the MTM group is removed to produce V. V is oxidized to the aldehyde with N-chlorosuccinimide (p.1059 MARC85 and FILL63). Compound VI is converted to a Grignard reagent and reacted with V to produce the alcohol VII. N-chlorosuccinimide is used to convert C_3 to a ketone; the ketone is converted to a gem-difluoride (compound VIII) with diethylaminosulfurtrifluoride (DAST) or one of the other reagents listed on p.809 of MARC85.

The THP group protecting the hydroxyl on C_3 is removed by mild acid aqueous hydrolysis (p.689 CARE90); the hydroxyl is converted to the chloride with PCl_5 or other suitable reagent (such as those listed on p.383 of MARC85) to yield compound IX. The MEM groups are then removed with non-aqueous zinc bromide (p.679 CARE90). C_4 is then oxidized to a keto group while C_1 is oxidized to a carboxylic acid with an appropriate oxidizing agent, such as $KMnO_4$ or CrO_3 (MARC85 p.1059 and p.1084) to yield compound X. The methyl ester of X is prepared by reaction with diazomethane (CARE90, p.134) and is reacted with potassium phthalimide (CARE90, p.132) to give XI (after treatment with hydrazine and hydrolysis of the methyl ester). XI is suitable for incorporation into peptide synthesis using Fmoc or tBoc chemistry.

The synthesis of XI does not establish definite chirality at C_2 or C_3; XI could be resolved into four components by, for example, chromatography over a chiral matrix such as an immobilized protein. Resolving XI into components of different chirality is preferred.

Alternatives in the synthesis include:

a) use of 2-butyl chloride Grignard reagent in place of 2-propyl chloride Grignard reagent in the first reaction. This change leads to synthesis of an analogue of ILE-ALA in which the linking -NH- group is replace by $-CF_2-$. Other alkyl chlorides may be used in place of 2-propyl chloride, leading to other dipeptide analogues in which the first amino acid is replaced.

b) replacing VI with XIII. This leads to synthesis of analogues of VAL-GLY or ILE-GLY. Other 1(O-MEM)-2-chloro compounds can be used in place of VI, leading to dipeptide analogues in which the second amino acid is different from ALA.

c) Using compounds XIV and XV in place of V and VI, one can prepare XVI having no F substituents.

d) Use of CH_3-CO_2F allows addition of -F and CH_3-COO- across a double bond (p.181 CARE90 and ref. 42 cite there). XVII can be obtained, for example, by dehydration of the Grignard adduct of XIV and XV. Addition of CH_3-CO_2F produces XVIII which can be converted to the monofluoro derivative of XI.

e) Closely related chemistry may be used to produce compounds having an additional $-CH_2-$ between C_2 and C_3 of XI.

Example 6

Figure 13 shows compounds involved in a hypothetical synthesis of dipeptide analogues that contain boron in place of carbonyl carbon. These analogues are used in Class I and Class

II inhibitors. Compound XXXI was reported by Matteson et al. (Organometallics 3:1284ff (1984) (MATT84)). XXXI is transesterified to give the isopropyl ester, XXXII. XXXIII is the MOM protected derivative of 1 hydroxy-2-methyl-3-chloropropane; XXXIII is reacted with lithium and the lithium derivative is reacted with XXXII to give XXXIV. The MOM group is removed, the free alcohol is oxidized to the aldehyde with N-chlorosuccinimide and then to the carboxylic acid with CrO_3. The free dipeptide analogue is shown as XXXV.

Example 7

Figure 14 shows compounds involved in a hypothetical synthesis of a molecule containing a boronic acid group. The boronic acid group is positioned so that, when R_3 occupies the S1' site, it occupies the site of the carbonyl carbon of residue P1. Compound XLI is readily prepared when R3 is -H, $-CH_3$, ethyl, etc. Matteson et al. MATT84 reports use of XLII. XLII imposed a particular chirality on XXXI (Figure 13). Reaction of XLII with XLI will produce XLIII. It is likely that the product will predominantly have one chirality at C_2. It is not known whether the chirality will be as shown in Figure 14 or the opposite. XLIV is obtained by removal of the MOM group and oxidation of the primary hydroxyl at C_1 to the carboxylic acid. XLIV can be coupled to free amines using N,N'-dicyclohexylcarbodiimide (DCC). As XLIV has no amine groups, XLIV is a chain terminator.

The R_1 linkers used in Class II inhibitors can be synthesized by standard methods as found in CARE90, MARC85, and other sources.

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Table 13: BPTI Homologues (1-19)

R #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
-3	-	-	-	F	-	-	-	-	-	-	-	-	-	-	-	-	Z	-	-
-2	-	-	-	Q	T	-	-	-	-	-	-	Q	-	-	-	H	G	Z	-
-1	-	-	-	T	E	-	-	-	-	-	-	P	-	-	-	D	D	G	-
1	R	R	R	P	R	R	R	R	R	R	R	L	A	R	R	R	K	R	A
2	P	P	P	P	P	P	P	P	P	P	P	R	A	P	P	P	R	P	A
3	D	D	D	D	D	D	D	D	D	D	D	K	K	D	R	T	D	S	K
4	F	F	F	L	F	F	F	F	F	F	F	L	Y	F	F	F	I	F	Y
5	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
6	L	L	L	Q	L	L	L	L	L	L	L	I	K	E	E	N	R	N	K
7	E	E	E	L	E	E	E	E	E	E	E	L	L	L	L	L	L	L	L
8	P	P	P	P	P	P	P	P	P	P	P	H	P	P	P	P	P	P	P
9	P	P	P	Q	P	P	P	P	P	P	P	R	L	A	A	P	P	A	V
10	Y	Y	Y	A	Y	Y	Y	Y	Y	Y	Y	N	R	E	E	E	E	E	R
11	T	T	T	R	T	T	T	T	T	T	T	P	I	T	T	S	Q	T	Y
12	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
13	P	P	P	P	P	P	P	P	P	P	P	R	P	L	L	R	P	P	P
14	C	T	A	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
15	K	K	K	K	K	V	G	A	L	I	K	Y	K	K	K	R	K	K	K
16	A	A	A	A	A	A	A	A	A	A	A	Q	R	A	A	G	G	A	K
17	R	R	R	A	A	R	R	R	R	R	R	K	K	Y	R	H	R	S	K
18	I	I	I	L	M	I	I	I	I	I	I	I	I	I	I	I	L	I	F
19	I	I	I	L	I	I	I	I	I	I	I	P	P	R	R	R	P	R	P
20	R	R	R	R	R	R	R	R	R	R	R	A	S	S	S	R	R	Q	S
21	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	F	F	F	F	I	Y	Y	F
22	F	F	F	F	F	F	F	F	F	F	F	Y	Y	H	H	Y	F	Y	Y
23	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
24	N	N	N	N	N	N	N	N	N	N	N	N	K	N	N	N	N	N	N
25	A	A	A	S	A	A	A	A	A	A	A	Q	W	L	R	L	P	S	W
26	K	K	K	T	K	K	K	K	K	K	K	K	K	A	A	E	A	K	K
27	A	A	A	S	A	A	A	A	A	A	A	K	A	A	A	S	S	S	A
28	G	G	G	N	G	G	G	G	G	G	G	K	K	Q	Q	N	R	G	K
29	L	L	L	A	F	L	L	L	L	L	L	Q	Q	Q	Q	K	M	G	Q
30	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C

R #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
31	Q	Q	Q	E	E	Q	Q	Q	Q	Q	Q	E	L	L	L	K	E	Q	L
32	T	T	T	P	T	T	T	T	T	T	T	G	P	Q	E	V	S	Q	P
33	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F
34	V	V	V	T	V	V	V	V	V	V	V	T	D	I	I	F	I	I	N
35	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	W	Y	Y	Y	Y	Y	Y	Y
36	G	G	G	G	G	G	G	G	G	G	G	S	S	G	G	G	G	G	S
37	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
38	C	T	A	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
39	R	R	R	Q	R	R	R	R	R	R	R	G	G	G	G	G	K	R	G
40	A	A	A	G	A	A	A	A	A	A	A	G	G	G	G	G	G	G	G
41	K	K	K	N	K	K	K	K	K	K	K	N	N	N	N	N	N	N	N
42	R	R	R	N	S	R	R	R	R	R	R	S	A	A	A	A	K	Q	A
43	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
44	N	N	N	N	N	N	N	N	N	N	N	R	R	R	R	N	N	R	R
45	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F
46	K	K	K	E	K	K	K	K	K	K	K	K	K	K	K	E	K	D	K
47	S	S	S	T	S	S	S	S	S	S	S	T	T	T	T	T	T	T	T
48	A	A	A	T	A	A	A	A	A	A	A	I	I	I	I	R	K	T	I
49	E	E	E	E	E	E	E	E	E	E	E	E	E	D	D	D	A	Q	E
50	D	D	D	M	D	D	D	D	D	D	D	E	E	E	E	E	E	Q	E
51	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
52	M	M	M	L	M	M	M	M	M	M	E	R	R	R	H	R	V	Q	R
53	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	E	R	G	R
54	T	T	T	I	T	T	T	T	T	T	T	T	T	T	T	T	A	V	T
55	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
56	G	G	G	E	G	G	G	G	G	G	G	I	V	V	V	G	R	V	V
57	G	G	G	P	G	G	G	G	G	G	G	R	G	G	G	G	P	-	G
58	A	A	A	P	A	A	A	A	A	A	A	K	-	-	-	K	P	-	-
59	-	-	-	Q	-	-	-	-	-	-	-	-	-	-	-	-	E	-	-
60	-	-	-	Q	-	-	-	-	-	-	-	-	-	-	-	-	R	-	-
61	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	P	-	-
62	-	-	-	D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
63	-	-	-	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
64	-	-	-	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 13, Continued (BPTI Homologues 20-35)

R #	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
-5	-	-	-	-	-	-	-	-	-	-	-	-	-	D	-	-
-4	-	-	-	-	-	-	-	-	-	-	-	-	-	E	-	-
-3	-	-	-	-	-	-	-	-	-	-	-	-	T	P	-	-
-2	Z	-	L	Z	R	K	-	-	-	R	R	-	E	T	-	-
-1	P	-	Q	D	D	N	-	-	-	Q	K	-	R	T	-	-
1	R	R	H	H	R	R	I	K	T	R	R	R	G	D	K	T
2	R	P	R	P	P	P	N	E	V	H	H	P	F	L	A	V
3	K	Y	T	K	K	T	G	D	A	R	P	D	L	P	D	E
4	L	A	F	F	F	F	D	S	A	D	D	F	D	I	S	A
5	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
6	I	E	K	Y	Y	N	E	Q	N	D	D	L	T	E	Q	N
7	L	L	L	L	L	L	L	L	L	K	K	E	S	Q	L	L
8	H	I	P	P	P	L	P	G	P	P	P	P	P	A	D	P
9	R	V	A	A	A	P	K	Y	V	P	P	P	P	FG	Y	I
10	N	A	E	D	D	E	V	S	I	D	D	Y	V	D	S	V
11	P	A	P	P	P	T	V	A	R	K	T	T	T	A	Q	Q
12	G	G	G	G	G	G	G	G	G	G	K	G	G	G	G	G
13	R	P	P	R	R	R	P	P	P	N	I	P	P	L	P	P
14	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
15	Y	M	K	K	L	N	R	M	R	-	-	K	R	F	L	R
16	D	F	A	A	A	A	A	G	A	G	Q	A	A	G	G	A
17	K	F	S	H	Y	L	R	M	F	P	T	K	G	Y	L	F
18	I	I	I	I	M	I	F	T	I	V	V	M	F	M	F	I
19	P	S	P	P	P	P	P	S	Q	R	R	I	K	K	K	Q
20	A	A	A	R	R	A	R	R	L	A	A	R	R	L	R	L
21	F	F	F	F	F	F	Y	Y	W	F	F	Y	Y	Y	Y	W
22	Y	Y	Y	Y	Y	Y	Y	F	A	Y	Y	F	N	S	F	A
23	Y	Y	Y	Y	Y	Y	Y	Y	F	Y	Y	Y	Y	Y	Y	F
24	N	S	N	D	N	N	N	N	D	D	K	N	N	N	N	D
25	Q	K	W	S	P	S	S	G	A	T	P	A	T	Q	G	A
26	K	G	A	A	A	H	S	T	V	R	S	K	R	E	T	V
27	K	A	A	S	S	L	S	S	K	L	A	A	T	T	S	K
28	K	N	K	N	N	H	K	M	G	K	K	G	K	K	M	G

R #	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
29	Q	K	K	K	K	K	R	A	K	T	R	F	Q	N	A	K
30	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
31	E	Y	Q	N	E	Q	E	E	V	K	V	E	E	E	E	V
32	R	P	L	K	K	K	K	T	L	A	Q	T	P	E	T	R
33	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F
34	D	T	H	I	I	N	I	Q	P	Q	R	V	K	I	L	S
35	W	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
36	S	S	G	G	G	G	G	G	G	R	G	G	G	G	G	G
37	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
38	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
39	G	R	K	P	R	G	G	M	Q	D	D	K	K	Q	M	K
40	G	G	G	G	G	G	G	G	G	G	G	A	G	G	G	G
41	N	N	N	N	N	N	N	N	N	D	D	K	N	N	N	N
42	S	A	A	A	A	A	A	G	G	H	H	S	G	D	L	G
43	N	N	N	N	N	N	N	N	N	G	G	N	N	N	N	N

Table 13, continued

R #	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
44	R	R	R	N	N	N	N	N	K	N	N	N	R	R	N	K
45	F	F	F	F	F	F	F	F	F	F	F	F	Y	F	F	F
46	K	K	S	K	K	K	H	V	Y	K	K	R	K	S	L	Y
47	T	T	T	T	T	T	T	T	S	T	S	S	S	T	S	S
48	I	I	I	W	W	I	L	E	E	E	D	A	E	L	Q	Q
49	E	E	E	D	D	D	E	K	K	T	H	E	Q	A	K	K
50	E	E	K	E	E	E	E	E	E	L	L	D	D	E	E	E
51	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
52	R	R	R	R	R	Q	E	L	R	R	R	M	L	E	L	K
53	R	R	H	Q	H	R	K	Q	E	C	C	R	D	Q	Q	E
54	T	T	A	T	T	T	V	T	Y	E	E	T	A	K	T	Y
55	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
56	I	V	V	G	V	A	G	R	G	L	E	G	S	I	R	G
57	G	V	G	A	A	A	V	-	V	V	L	G	G	N	-	I
58	-	-	-	S	S	K	R	-	P	Y	Y	A	F	-	-	P
59	-	-	-	A	G	Y	S	-	G	P	R	-	-	-	-	G
60	-	-	-	-	I	G	-	-	D	-	-	-	-	-	-	E
61	-	-	-	-	-	-	-	-	E	-	-	-	-	-	-	A

Table 13, continued (Homologues 36-40)

R #	36	37	38	39	40
-5	-	-	-	-	-
-4	-	-	-	-	-
-3	-	-	-	-	-
-2	-	-	-	-	-
-1	-	Z	-	-	-
1	R	R	R	R	R
2	P	P	P	P	P
3	D	D	D	D	D
4	F	F	F	F	F
5	C	C	C	C	C
6	L	L	L	L	L
7	E	E	E	E	E
8	P	P	P	P	P
9	P	P	P	P	P
10	Y	Y	Y	Y	Y
11	T	T	T	T	T
12	G	G	G	G	G
13	P	P	P	P	P
14	C	C	C	C	C
15	R	K	K	K	K
16	A	A	A	A	A
17	R	R	R	R	K
18	I	M	I	M	M
19	I	I	I	I	I
20	R	R	R	R	R
21	Y	Y	Y	Y	Y
22	F	F	F	F	F
23	Y	Y	Y	Y	Y
24	N	N	N	N	N
25	A	A	A	A	A
26	K	K	K	K	K
27	A	A	A	A	A

	28	G	G	G	G	G
	29	L	L	L	L	F
	30	C	C	C	C	C
	31	Q	Q	Q	Q	E
5	32	T	P	P	P	T
	33	F	F	F	F	F
	34	V	V	V	V	V
	35	Y	Y	Y	Y	Y
	36	G	G	G	G	G
10	37	G	G	G	G	G
	38	C	C	C	C	C
	39	R	R	R	R	K
	40	A	A	A	A	A
	41	K	K	K	K	K
15	42	R	S	R	R	S
	43	N	N	N	N	N

Table 13, continued

R #	36	37	38	39	40
44	N	N	N	N	N
45	F	F	F	F	F
46	K	K	K	K	R
47	S	S	S	S	S
48	A	A	S	A	A
49	E	E	E	E	E
50	D	D	D	D	D
51	C	C	C	C	C
52	E	M	M	M	M
53	R	R	R	R	R
54	T	T	T	T	T
55	C	C	C	C	C
56	G	G	G	G	G
57	G	G	G	G	G
58	A	A	A	A	A
59	-	-	-	-	-
60	-	-	-	-	-
61	-	-	-	-	-

Legend to Table 13

1 BPTI
2 Engineered BPTI From MARK87
3 Engineered BPTI From MARK87
4 Bovine Colostrum (DUFT85)
5 Bovine Serum (DUFT85)
6 Semisynthetic BPTI, TSCH87
7 Semisynthetic BPTI, TSCH87
8 Semisynthetic BPTI, TSCH87
9 Semisynthetic BPTI, TSCH87
10 Semisynthetic BPTI, TSCH87
11 Engineered BPTI, AUER87
12 Dendroaspis polylepis polylepis (Black mamba) venom I (DUFT85)
13 Dendroaspis polylepis polylepis (Black Mamba) venom K (DUFT85)
14 Hemachatus hemachates (Ringhals Cobra) HHV II (DUFT85)
15 Naja nivea (Cape cobra) NNV II (DUFT85)
16 Vipera russelli (Russel's viper) RVV II (TAKA74)
17 Red sea turtle egg white (DUFT85)
18 Snail mucus (Helix pomania) (WAGN78)
19 Dendroaspis angusticeps (Eastern green mamba) C13 S1 C3 toxin (DUFT85)
20 Dendroaspis angusticeps (Eastern Green Mamba) C13 S2 C3 toxin (DUFT85)
21 Dendroaspis polylepis polylepes (Black mamba) B toxin (DUFT85)
22 Dendroaspis polylepis polylepes (Black Mamba) E toxin (DUFT85)
23 Vipera ammodytes TI toxin (DUFT85)
24 Vipera ammodytes CTI toxin (DUFT85)
25 Bungarus fasciatus VIII B toxin (DUFT85)
26 Anemonia sulcata (sea anemone) 5 II (DUFT85)
27 Homo sapiens HI-8e "inactive" domain (DUFT85)
28 Homo sapiens HI-8t "active" domain (DUFT85)
29 beta bungarotoxin B1 (DUFT85)
30 beta bungarotoxin B2 (DUFT85)
31 Bovine spleen TI II (FIOR85)
32 Tachypleus tridentatus (Horseshoe crab) hemocyte inhibitor (NAKA87)
33 Bombyx mori (silkworm) SCI-III (SASA84)
34 Bos taurus (inactive) BI-14
35 Bos taurus (active) BI-8
36:Engineered BPTI (KR15, ME52): Auerswald '88, Biol Chem Hoppe-Seyler, 369 Supplement, pp27-35.
37:Isoaprotinin G-1: Siekmann, Wenzel, Schroder, and Tschesche '88, Biol Chem Hoppe-Seyler, 369:157-163.
38:Isoaprotinin 2: Siekmann, Wenzel, Schroder, and Tschesche '88, Biol Chem Hoppe-Seyler, 369:157-163.
39:Isoaprotinin G-2: Siekmann, Wenzel, Schroder, and

Tschesche '88, Biol Chem Hoppe-Seyler, 369:157-163.
40:Isoaprotinin 1: Siekmann, Wenzel, Schroder, and Tschesche '88, Biol Chem Hoppe-Seyler, 369:157-163.

Notes :

a) both beta bungarotoxins have residue 15 deleted.
b) B. mori has an extra residue between C5 and C14; we have assigned F and G to residue 9.
c) all natural proteins have C at 5, 14, 30, 38, 50, & 55.
d) all homologues have F33 and G37.
e) extra C's in bungarotoxins form interchain cystine bridges

Table 15: Frequency of Amino Acids at Each Position in BPTI and 58 Homologues

Res. Id.	Different AAs	Contents	First
-5	2	-58 D	-
-4	2	-58 E	-
-3	5	-55 P T Z F	-
-2	10	-43 R3 Z3 Q3 T2 E G H K L	-
-1	11	-41 D4 P3 R2 T2 Q2 G K N Z E	-
1	13	R35 K6 T4 A3 H2 G2 L M N P I D -	R
2	10	P35 R6 A4 V4 H3 E3 N F I L	P
3	11	D32 K8 S4 A3 T3 R2 E2 P2 G L Y	D
4	9	F34 A6 D4 L4 S4 Y3 I2 W V	F
5	1	C59	C
6	13	L25 N7 E6 K4 Q4 I3 D2 S2 Y2 R F T A	L
7	7	L28 E25 K2 F Q S T	E
8	10	P46 H3 D2 G2 E I K L A Q	P
9	12	P30 A9 I4 V4 R3 Y3 L F Q H E K	P
9a	2	-58 G	-
10	9	Y24 E8 D8 V6 R3 S3 A3 N3 I	Y
11	11	T31 Q8 P7 R3 A3 Y2 K S D V I	T
12	2	G58 K	G
13	5	P45 R7 L4 I2 N	P
14	3	C57 A T	C
15	12	K22 R12 L7 V6 Y3 M2 -2 N I A F G	K
16	7	A41 G9 F2 D2 K2 Q2 R	A
17	14	R19 L8 K7 F5 M4 Y4 H2 A2 S2 G2 I N T P	R
18	8	I41 M7 F4 L2 V2 E T A	I
19	10	I24 P12 R8 K5 S4 Q2 L N E T	I
20	5	R39 A8 L6 S5 Q	R
21	5	Y35 F17 W5 I L	Y
22	6	F32 Y18 A5 H2 S N	F
23	2	Y52 F7	Y
24	4	N47 D8 K3 S	N
25	13	A29 S6 Q4 G4 W4 P3 T2 L2 R N K V I	A
26	11	K31 A9 T5 S3 V3 R2 E2 G H F Q	K
27	8	A32 S11 K5 T4 Q3 L2 I E	A
28	7	G32 K13 N5 M4 Q2 R2 H	G
29	10	L22 K13 Q11 A5 F2 R2 N G M T	L
30	2	C58 A	C
31	10	Q25 E17 L5 V5 K2 N A R I Y	Q
32	11	T25 P11 K4 Q4 L4 R3 E3 G2 S A V	T
33	1	F59	F
34	13	V24 I10 T5 N3 Q3 D3 K3 F2 H2 R S P L	V
35	2	Y56 W3	Y
36	3	G50 S8 R	G
37	1	G59	G
38	3	C57 A T	C

Table 15: Frequency of Amino Acids at Each Position in BPTI and 58 Homologues (continued)

Res. Id.	Different AAs	Contents	First
39	9	R25 G13 K6 Q4 E3 M3 L2 D2 P	R
40	2	G35 A24	A
41	3	N33 K24 D2	K
42	12	R22 A12 G8 S6 Q2 H2 N2 M D E K L	R
43	2	N57 G2	N
44	3	N40 R14 K5	N
45	2	F58 Y	F
46	11	K39 Y5 E4 S2 V2 D2 R H T A L	K
47	2	S36 T23	S
48	11	A23 I11 E6 Q6 L4 K2 T2 W2 S D R	A
49	8	E37 K8 D6 Q3 A2 P H T	E
50	7	E27 D25 K2 L2 M Q Y	D
51	2	C58 A	C
52	9	M17 R15 E8 L7 K6 Q2 T2 H V	M
53	11	R37 E6 Q5 K2 C2 H2 A N G D W	R
54	8	T41 Y5 A4 V3 I2 E2 M K	T
55	1	C59	C
56	10	G33 V9 R5 I4 E3 L A S T K	G
57	12	G34 V6 -5 A3 R2 I2 P2 D K S L N	G
58	10	A25 -15 P7 K3 S2 Y2 G2 F D R	A

Legend for Table 15

1 BPTI
2 synthetic BPTI, Tan & Kaiser, biochem. 16(8)1531-41
3 Semisynthetic BPTI, TSCH87
4 Semisynthetic BPTI, TSCH87
5 Semisynthetic BPTI, TSCH87
6 Semisynthetic BPTI, TSCH87
7 Semisynthetic BPTI, TSCH87
8 Engineered BPTI, AUER87
9 BPTI Auerswald &al GB 2 208 511A
10 BPTI Auerswald &al GB 2 208 511A
11 Engineered BPTI From MARK87
12 Engineered BPTI From MARK87
13BPTI(KR15,ME52): Auerswald '88, Biol Chem Hoppe-Seyler, 369 Suppl, pp27-35.
14BPTI CA30/CA51 Eigenbrot &al, Protein Engineering 3(7)591-598 ('90)
15Isoaprotinin 2 Siekmann et al '88, Biol Chem Hoppe-Seyler, 369:157-163.
16Isoaprotinin G-2: Siekmann et al '88, Biol Chem Hoppe-Seyler, 369:157-163.
17 BPTI Engineered, Auerswald &al GB 2 208 511A
18 BPTI Engineered, Auerswald &al GB 2 208 511A
19 BPTI Engineered, Auerswald &al GB 2 208 511A
20Isoaprotinin G-1 Siekmann &al '88, Biol Chem Hoppe-Seyler, 369:157-163.
21 BPTI Engineered, Auerswald &al GB 2 208 511A
22 BPTI Engineered, Auerswald &al GB 2 208 511A
23 Bovine Serum (in Dufton '85)
24 Bovine spleen TI II (FIOR85)
25 Snail mucus (Helix pomatia) (WAGN78)
26Hemachatus hemachates (Ringhals Cobra) HHV II (in Dufton '85)
27 Red sea turtle egg white (in Dufton '85)
28 Bovine Colostrum (in Dufton '85)
29 Naja nivea (Cape cobra) NNV II (in Dufton '85)
30 Bungarus fasciatus VIII B toxin (in Dufton '85)
31 Vipera ammodytes TI toxin (in Dufton '85)
32 Porcine ITI domain 1, (in CREI87)
33Human Alzheimer's beta APP protease inhibitor, (SHIN90)
34 Equine ITI domain 1, in Creighton & Charles
35 Bos taurus (inactive) BI-8e (ITI domain 1)
36Anemonia sulcata (sea anemone) 5 II (in Dufton '85)
37Dendroaspis polylepis polylepes (Black Mamba) E toxin (in Dufton '85)
38Vipera russelli (Russel's viper) RVV II (TAKA74)
39Tachypleus tridentatus (Horseshoe crab) hemocyte inhibitor (NAKA87)
40 LACI 2 (Factor Xa) (WUNT88)
41 Vipera ammodytes CTI toxin (in Dufton '85)

Identification codes for Tables 14 and 15

42Dendroaspis polylepis polylepis (Black Mamba) venom K (in Dufton '85)
43Homo sapiens HI-8e "inactive" domain (in Dufton '85)
44Green Mamba toxin K, (in CREI87)
45Dendroaspis angusticeps (Eastern green mamba) C13 S1 C3 toxin (in Dufton '85)
46 LACI 3
47 Equine ITI domain 2, (CREI87)
48 LACI 1 (VIIa)
49Dendroaspis polylepis polylepes (Black mamba) B toxin (in Dufton '85)
50 Porcine ITI domain 2, Creighton and Charles
51Homo sapiens HI-8t "active" domain (in Dufton '85)
52 Bos taurus (active) BI-8t
53Trypstatin Kito &al ('88) J Biol Chem 263(34)18104-07
54Dendroaspis angusticeps (Eastern Green Mamba) C13 S2 C3 toxin (in Dufton '85)
55Green Mamba I venom Creighton & Charles '87 CSHSQB 52:511-519.
56 beta bungarotoxin B2 (in Dufton '85)
57Dendroaspis polylepis polylepis (Black mamba) venom I (in Dufton '85)
58 beta bungarotoxin B1 (in Dufton '85)
59 Bombyx mori (silkworm) SCI-III (SASA84)

Table 61: Variability of Naturally-occuring Kunitz domains

Res. Id.	Different AAs	Contents											BPTI
1	12	R16	K6	T4	A3	H2	G2	M	N	P	I	L	-	R
2	9	P18	R6	A4	V4	E3	N	F	H	I				P
3	10	D14	K8	S4	A3	T3	G2	E2	L	R	Y			D
4	9	F17	A6	L4	S4	Y3	D2	V	W	I				F
5	1	C39												C
6	12	L7	N7	E6	K4	Q4	I3	S2	Y2	R	F	T	A	L
7	5	L29	E7	F	S	T								E
8	9	P27	H3	D2	G2	I	K	L	E	Q				P
9	11	A10	P10	I4	V4	R3	Y3	H	Q	E	K	L		P
10	9	E8	V6	Y6	D5	A4	S3	N3	R3	I				Y
11	11	T12	Q8	P7	R3	A2	Y2	K	S	D	V	I		T
12	1	G39												G
13	4	P27	R7	L4	I									P
14	1	C39												C
15	6	K18	R11	L4	Y3	M2	N							K
16	7	A25	G7	D2	K2	F	Q	R						A
17	12	K7	R7	F5	M4	H3	Y3	A2	G2	S2	L2	N	I	R
18	8	I23	M6	F4	L2	K	A	E	T					I
19	10	P13	I6	R6	K4	S4	Q2	L	N	E	T			I
20	5	R21	A7	L5	S5	Q								R
21	5	F16	Y16	W5	I	L								Y
22	5	Y17	F14	A5	H2	N								F

23	2	Y32	F7											Y
24	4	N29	D7	K2	S									N
25	13	A11	S6	G4	W4	Q3	K2	L2	P2	R	I	T	V	N	A
26	12	K13	A9	T5	V3	S2	H	D	Q	R	E	F	G		K
27	8	A13	S12	K5	Q3	T3	I	E	L						A
28	7	G14	K10	N5	M4	H2	Q2	R2							G
29	8	K13	Q11	A5	L4	F2	R2	G	M						L
30	1	C39													C
31	10	E16	Q8	L5	V4	A	N	I	R	K	Y				Q
32	10	P11	T7	K5	L4	R3	Q3	E2	G2	S	V				T
33	1	F39													F
34	12	I10	V6	T5	N3	D3	K3	Q2	H2	F2	S	P	L		V
35	2	Y36	W3												Y
36	2	G31	S8												G
37	1	G39													G
38	1	C39													C
39	8	G14	R9	K6	Q3	M3	L2	E	P						R

Table 61: Variability of Naturally-occuring Kunitz domains (continued)

Res. Id.	Different AAs	Contents									BPTI
40	2	G33	A6								A
41	2	N33	K6								K
42	10	A13	G8	S6	R4	N2	Q2	E	K	L	M	R
43	1	N39									N
44	3	N20	R14	K5							N
45	2	F38	Y								F
46	11	K19	Y5	E4	R2	V2	D2	H	S	T	A	L	K
47	2	T22	S17								S
48	10	I12	Q6	A5	E5	L3	K2	T2	W2	R	S	A
49	6	E19	K8	D7	Q3	P	A				E
50	6	E27	D7	K2	M	Q	Y				D
51	1	C39									C
52	9	R13	M7	L7	K6	Q2	N	H	E	V	M
53	10	R20	E6	Q4	H2	K2	A	N	G	D	W	R
54	6	T24	Y5	A4	V3	I2	M				T
55	1	C39									C
56	9	G15	V10	R5	I3	E2	A	S	T	K	G
57	10	G17	V5	-5	A3	R2	I2	P2	S	D	K	G
58	9	-15	P7	A7	K3	S2	G2	R	F	D	A

Table 62: Kunitz sequences used in compilation of Table 61

1BPTI
2Isoaprotinin 2 (SIEK88)
3Isoaprotinin G-2 (SIEK88)
4Isoaprotinin G-1 (SIEK88)
5Bovine Serum (in DUFT85)
6Bovine spleen TI II (FIOR85)
7Snail mucus (*Helix pomatia*) (WAGN78)
8*Hemachatus hemachates* (Ringhals Cobra) HHV II (in DUFT85)
9Red sea turtle egg white (in DUFT85)
10Bovine Colostrum (in DUFT85)
11*Naja nivea* (Cape cobra) NNV II (in DUFT85)
12*Bungarus fasciatus* VIII B toxin (in DUFT85)
13*Vipera ammodytes* TI toxin (in DUFT85)
14Porcine ITI domain 1, (in CREI87)
15Human Alzheimer's β APP protease inhibitor (SINH90)
16Equine ITI domain 1 (in CREI87)
17*Bos taurus* (inactive) BI-8e (ITI domain 1) (in CREI87)
18*Anemonia sulcata* (sea anemone) 5 II (in DUFT85)
19*Dendroaspis polylepis polylepes* (Black Mamba) E toxin (in DUFT85)
20*Vipera russelli* (Russel's viper) RVV II (TAKA74)
21*Tachypleus tridentatus* (Horseshoe crab) hemocyte inhibitor (NAKA87)
22LACI 2 (Factor Xa) (WUNT88)
23*Vipera ammodytes* CTI toxin (in DUFT85)
24*Naja naja naja* venom (SHAF90)
25*Dendroaspis polylepis polylepis* (Black Mamba) venom K (in DUFT85)
26*Homo sapiens* HI-8e "inactive" domain (in DUFT85)
27Green Mamba toxin K, (in CREI87)
28*Dendroaspis angusticeps* (Eastern green mamba) C13 S1 C3 toxin (in DUFT85)
29LACI 3 (WUNT88)
30Equine ITI domain 2 (in CREI87)
31LACI 1 (VIIa) (GIRA90)
32*Dendroaspis polylepis polylepes* (Black mamba) B toxin (in DUFT85)
33Porcine ITI domain 2 (in CREI887)
34*Homo sapiens* HI-8t "active" domain (in DUFT85)
35*Bos taurus* (active) BI-8t (in CREI87)
36Trypstatin (KITO88)
37*Dendroaspis angusticeps* (Eastern Green Mamba) C13 S2 C3 toxin (in DUFT85)
38Green Mamba I venom (in CREI87)
39*Dendroaspis polylepis polylepis* (Black mamba) venom I (in DUFT85)

Table 63: Histogram of (Number of residues having given variability) *vs.* Variability

N different	58 locations	Core 51 sites
1	10	10
2	7	7
3	1	1
4	2	2
5	4	4
6	4	4
7	2	2
8	4	4
9	7	5
10	8	7
11	3	3
12	5	4
13	1	1

Table 64: Citations for Table of Naturally-Occurring Kunitz Domains (Table 62)

CREI87Creighton & Charles (1987) *Cold Spring Harbor Symp Quant Biol* **52**:511-519.
DUFT85Dufton (1985) *Eur J Biochem* **153**:647-654.
FIOR85Fioretti *et al.* (1985) *J Biol Chem* **260**:11451-11455.
GIRA90 Girard *et al.* (1990) *Science* **248**:1421-24.
KITO88 Kito *et al.* (1988) *J Biol Chem* **263**(34)18104-07
NAKA87Nakamura *et al.* (1987) *J Biochem* **101**:1297-1306.
SHAF90 Shafqat *et al.* (1990) *Eur J Biochem* **194**:337-341.
SIEK88 Siekmann *et al.*(1988) *Biol Chem Hoppe-Seyler*, **369**:157-163.
TAKA74Takahashi *et al.* (1974) *J Biochem* **76**:721-733.
WAGN78Wagner *et al.* (1978) *Eur J Biochem* **89**:367-377.
WUNT88 Wun *et al.* (1988) *J Biol Chem* **263**:6001-4.

Table 65: Effects of mutations to Kunitz domains on binding to serine proteases.

Classes:
- A No major effect expected if molecular charge stays in range -1 to +1.
- B Major effects not xpected, but are more likely than in "A".
- C Residue in the binding interface; any change must be tested.
- X No substitution allowed.

Res. Id.	EpiNE1	Substitutions	Class
1	R	any	A
2	P	any	A
3	D	any	A
4	F	Y, W, L	B
5	C	C	X
6	L	non-proline	A
7	E	L, S, T, D, N, K, R	A
8	P	any	A
9	P	any	A
10	Y	non-proline prefr'd	B
11	T	any	C
12	G	must be G	X
13	P	any	C
14	C	C strongly preferred, any non-proline	C
15	I	V, A	C
16	A		C
17	F	L, I, M, Y, W, H, V	C
18	F	Y, W, H	C
19	P	any	C
20	R	non-proline prefr'd	C
21	Y	F & Y most prefr'd; W, I, L prefr'd; M, V allowed	C
22	F	Y & F most prefr'd; non-proline prefr'd	Y, F B
23	Y	Y & F strongly prefr'd	F ,Y B
24	N	non-proline prefr'd	A
25	A	any	A
26	K	any	A
27	A	any	A
28	G	non-proline prefr'd	A
29	L	non-proline prefr'd	A
30	C	must be C	X
31	Q	non-proline prefr'd	B
32	T	non-proline prefr'd	B
33	F	F very strongly prefr'd; Y possible	X
34	V	any	C
35	Y	Y most prefr'd; W prefr'd; F allowed	B

Table 65: Effects of mutations to Kunitz domains on binding to serine proteases. (continued)

Res. Id.	EpiNE1	Substitutions	Class
36	G	G strongly prefr'd; S, A prefr'd;	C
37	G	must be G so long as 38 is C	X
38	C	C strongly prefr'd	X
39	M	any	C
40	G	A,S,N,D,T,P	C
41	N	K,Q,S,D,R,T,A,E	C
42	G	any	C
43	N	must be N	X
44	N	S,K,R,T,Q,D,E	B
45	F	Y	B
46	K	any non-proline	B
47	ST, N, A, G		B
48	Aany	B	
49	E	any	A
50	D	any	A
51	C	must be C	X
52	M	any	A
53	R	any	A
54	T	any	A
55	C	must be C	X
56	G	any	A
57	G	any	A
58	A	any	A

prefr'd stands for preferred.

TABLE 203

Effect of pH on the Disociation of Bound BPTI-III MK and BPTI(K15L)-III MA Phage from Immobilized HNE

	BPTI-III MK		BPTI(K15L)-III MA	
pH	Total Plaque-Forming Units in Fraction	% of Input Phage	Total Plaque-Forming Units in Fraction	% of Input Phage
7.0	$5.0 \cdot 10^4$	$2 \cdot 10^{-3}$	$1.7 \cdot 10^5$	$3.2 \cdot 10^{-2}$
6.0	$3.8 \cdot 10^4$	$2 \cdot 10^{-3}$	$4.5 \cdot 10^5$	$8.6 \cdot 10^{-2}$
5.0	$3.5 \cdot 10^4$	$1 \cdot 10^{-3}$	$2.1 \cdot 10^6$	$4.0 \cdot 10^{-1}$
4.0	$3.0 \cdot 10^4$	$1 \cdot 10^{-3}$	$4.3 \cdot 10^6$	$8.2 \cdot 10^{-1}$
3.0	$1.4 \cdot 10^4$	$1 \cdot 10^{-3}$	$1.1 \cdot 10^6$	$2.1 \cdot 10^{-1}$
2.2	$2.9 \cdot 10^4$	$1 \cdot 10^{-3}$	$5.9 \cdot 10^4$	$1.1 \cdot 10^{-2}$
	Percentage of Input Phage Recovered = $8.0 \cdot 10^{-3}$		Percentage of Input Phage Recovered = 1.56	

The total input of BPTI-III MK phage was 0.030 ml x $(8.6 \cdot 10^{10}$ pfu/ml) = $2.6 \cdot 10^9$.

The total input of BPTI(K15L)-III MA phage was 0.030 ml x $(1.7 \cdot 10^{10}$ pfu/ml) = $5.2 \cdot 10^8$.

Given that the infectivity of BPTI(K15L)-III MA phage is 5 fold lower than that of BPTI-III MK phage, the phage inputs utilized above ensure that an equivalent number of phage particles are added to the immobilized HNE.

TABLES 207-208 (merged)

SEQUENCES OF THE EpiNE CLONES IN THE P1 REGION

CLONE IDENTIFIERS	SEQUENCE									
	1 3	1 4	1 5	1 6	1 7	1 8	1 9	2 0	2 1	
BPTI(comp. only)	P	C	K	A	R	I	I	R	Y	(BPTI)
	P	C	V	A	M	F	Q	R	Y	EpiNEα
	CCT.	TGC.	GTG.	GCT.	ATG.	TTC.	CAA.	CGC.	TAT	
3, 9, 16, 17, 18, 19	P	C	V	G	F	F	S	R	Y	EpiNE3
	CCT.	TGC.	GTC.	GGT.	TTC.	TTC.	TCA.	CGC.	TAT	
6	P	C	V	G	F	F	Q	R	Y	EpiNE6
	CCT.	TGC.	GTC.	GGT.	TTC.	TTC.	CAA.	CGC.	TAT	
7, 13, 14 15, 20	P	C	V	A	M	F	P	R	Y	EpiNE7
	CCT.	TGC.	GTC.	GCT.	ATG.	TTC.	CCA.	CGC.	TAT	
4	P	C	V	A	I	F	P	R	Y	EpiNE4
	CCT.	TGC.	GTC.	GCT.	ATC.	TTC.	CCA.	CGC.	TAT	
8	P	C	V	A	I	F	K	R	S	EpiNE8
	CCT.	TGC.	GTC.	GCT.	ATC.	TTC.	AAA.	CGC.	TCT	
1, 10 11, 12	P	C	I	A	F	F	P	R	Y	EpiNE1
	CCT.	TGC.	ATC.	GCT.	TTC.	TTC.	CCA.	CGC.	TAT	
5	P	C	I	A	F	F	Q	R	Y	EpiNE5
	CCT.	TGC.	ATC.	GCT.	TTC.	TTC.	CAA.	CGC.	TAT	
2	P	C	I	A	L	F	K	R	Y	EpiNE2
	CCT.	TGC.	ATC.	GCT.	TTG.	TTC.	AAA.	CGC.	TAT	

Table 209: DNA sequences and predicted amino acid sequences around the P1 region of BPTI analogues selected for binding to Cathepsin G.

Clone		P1										
	10	15	16	17	18	19	39	40	41	42	52	F
BPTI	TYR	LYS	ALA	ARG	ILE	ILE	ARG	ALA	LYS	ARG	MET	-
BRINK	TYR	PHE	ALA	PHE	ILE	ILE	ARG	ALA	LYS	ARG	GLU	-
EpiC 1	TYR	MET	GLY	PHE	SER	LYS	MET	GLY	ASN	GLY	MET	3/7
EpiC 7	TYR	MET	ALA	LEU	PHE	LYS	MET	GLY	ASN	GLY	MET	1/7
EpiC 8	ASN	PHE	ALA	ILE	THR	PRO	MET	GLY	ASN	GLY	MET	1/7
EpiC 10	TYR	MET	ALA	LEU	PHE	GLN	MET	GLY	ASN	GLY	MET	1/7
EpiC 20	TYR	MET	ALA	ILE	SER	PRO	MET	GLY	ASN	GLY	MET	1/7
EpiC 31	TYR	MET	ALA	ILE	SER	PRO	MET	GLY	ASN	GLY	MET	2/15
EpiC 32	TYR	MET	ALA	ILE	SER	PRO	GLU	ALA	LYS	ARG	MET	7/15
EpiC 33	TYR	MET	ASP	ILE	SER	PRO	MET	GLY	ASN	GLY	MET	1/15
EpiC 34	TYR	MET	ASP	ILE	SER	PRO	GLU	ALA	LYS	ARG	MET	4/15
EpiC 35	TYR	LEU	ASP	ILE	SER	PRO	GLU	ALA	LYS	ARG	MET	1/15

TABLE 211

Effects of antisera on phage infectivity

Phage (dilution of stock)	Incubation Conditions	pfu/ml	Relative Titer
MA-ITI $1.2 \cdot 10^{11}$ (10^{-1})	PBS	1.00	
	NRS	$6.8 \cdot 10^{10}$	0.57
	anti-ITI	$1.1 \cdot 10^{10}$	0.09
MA-ITI (10^{-3})	PBS	$7.7 \cdot 10^{8}$	1.00
	NRS	$6.7 \cdot 10^{8}$	0.87
	anti-ITI	$8.0 \cdot 10^{6}$	0.01
MA (10^{-1})	PBS	$1.3 \cdot 10^{12}$	1.00
	NRS	$1.4 \cdot 10^{12}$	1.10
	anti-ITI	$1.6 \cdot 10^{12}$	1.20
MA (10^{-3})	PBS	$1.3 \cdot 10^{10}$	1.00
	NRS	$1.2 \cdot 10^{10}$	0.92
	anti-ITI	$1.5 \cdot 10^{10}$	1.20

TABLE 212

Fractionation of EpiNE-7 and MA-ITI phage on hNE beads

Sample	EpiNE-7		MA-ITI	
	Total pfu in sample	Fraction of input	Total pfu in sample	Fraction of input
INPUT	$3.3 \cdot 10^{9}$	1.00	$3.4 \cdot 10^{11}$	1.00
Final TBS-TWEEN Wash	$3.8 \cdot 10^{5}$	$1.2 \cdot 10^{-4}$	$1.8 \cdot 10^{6}$	$5.3 \cdot 10^{-6}$
pH 7.0	$6.2 \cdot 10^{5}$	$1.8 \cdot 10^{-4}$	$1.6 \cdot 10^{6}$	$4.7 \cdot 10^{-6}$
pH 6.0	$1.4 \cdot 10^{6}$	$4.1 \cdot 10^{-4}$	$1.0 \cdot 10^{6}$	$2.9 \cdot 10^{-6}$
pH 5.5	$9.4 \cdot 10^{5}$	$2.8 \cdot 10^{-4}$	$1.6 \cdot 10^{6}$	$4.7 \cdot 10^{-6}$
pH 5.0	$9.5 \cdot 10^{5}$	$2.9 \cdot 10^{-4}$	$3.1 \cdot 10^{5}$	$9.1 \cdot 10^{-7}$
pH 4.5	$1.2 \cdot 10^{6}$	$3.5 \cdot 10^{-4}$	$1.2 \cdot 10^{5}$	$3.5 \cdot 10^{-7}$
pH 4.0	$1.6 \cdot 10^{6}$	$4.8 \cdot 10^{-4}$	$7.2 \cdot 10^{4}$	$2.1 \cdot 10^{-7}$
pH 3.5	$9.5 \cdot 10^{5}$	$2.9 \cdot 10^{-4}$	$4.9 \cdot 10^{4}$	$1.4 \cdot 10^{-7}$
pH 3.0	$6.6 \cdot 10^{5}$	$2.0 \cdot 10^{-4}$	$2.9 \cdot 10^{4}$	$8.5 \cdot 10^{-8}$
pH 2.5	$1.6 \cdot 10^{5}$	$4.8 \cdot 10^{-5}$	$1.4 \cdot 10^{4}$	$4.1 \cdot 10^{-8}$
pH 2.0	$3.0 \cdot 10^{5}$	$9.1 \cdot 10^{-5}$	$1.7 \cdot 10^{4}$	$5.0 \cdot 10^{-8}$
SUM*	$6.4 \cdot 10^{6}$	$3 \cdot 10^{-3}$	$5.7 \cdot 10^{6}$	$2 \cdot 10^{-5}$

* SUM is the total pfu (or fraction of input) obtained from all pH elution fractions

TABLE 213

Fractionation of EpiC-10 and MA-ITI phage on Cat-G beads

	EpiC-10		MA-ITI	
Sample	Total pfu in sample	Fraction of input	Total pfu in sample	Fraction of input
INPUT	$5.0 \cdot 10^{11}$	1.00	$4.6 \cdot 10^{11}$	1.00
Final TBS-TWEEN Wash	$1.8 \cdot 10^{7}$	$3.6 \cdot 10^{-5}$	$7.1 \cdot 10^{6}$	$1.5 \cdot 10^{-5}$
pH 7.0	$1.5 \cdot 10^{7}$	$3.0 \cdot 10^{-5}$	$6.1 \cdot 10^{6}$	$1.3 \cdot 10^{-5}$
pH 6.0	$2.3 \cdot 10^{7}$	$4.6 \cdot 10^{-5}$	$2.3 \cdot 10^{6}$	$5.0 \cdot 10^{-6}$
pH 5.5	$2.5 \cdot 10^{7}$	$5.0 \cdot 10^{-5}$	$1.2 \cdot 10^{6}$	$2.6 \cdot 10^{-6}$
pH 5.0	$2.1 \cdot 10^{7}$	$4.2 \cdot 10^{-5}$	$1.1 \cdot 10^{6}$	$2.4 \cdot 10^{-6}$
pH 4.5	$1.1 \cdot 10^{7}$	$2.2 \cdot 10^{-5}$	$6.7 \cdot 10^{5}$	$1.5 \cdot 10^{-6}$
pH 4.0	$1.9 \cdot 10^{6}$	$3.8 \cdot 10^{-6}$	$4.4 \cdot 10^{5}$	$9.6 \cdot 10^{-7}$
pH 3.5	$1.1 \cdot 10^{6}$	$2.2 \cdot 10^{-6}$	$4.4 \cdot 10^{5}$	$9.6 \cdot 10^{-7}$
pH 3.0	$4.8 \cdot 10^{5}$	$9.6 \cdot 10^{-7}$	$3.6 \cdot 10^{5}$	$7.8 \cdot 10^{-7}$
pH 2.5	$2.0 \cdot 10^{5}$	$4.0 \cdot 10^{-7}$	$2.7 \cdot 10^{5}$	$5.9 \cdot 10^{-7}$
pH 2.0	$2.4 \cdot 10^{5}$	$4.8 \cdot 10^{-7}$	$3.2 \cdot 10^{5}$	$7.0 \cdot 10^{-7}$
SUM*	$9.9 \cdot 10^{7}$	$2 \cdot 10^{-4}$	$1.4 \cdot 10^{7}$	$3 \cdot 10^{-5}$

* SUM is the total pfu (or fraction of input) obtained from all pH elution fractions

AFFINITY CLASS	ESTIMATED K_D	FRACTION OF INPUT BOUND	pH ELUTION MAXIMUM	PROTEIN
WEAK	$K_D > 10^{-8}$ M	<0.005%>	pH 6.0	ITI-D1
MODERATE	10^{-8} M to 10^{-9} M	0.01% to 0.03%	pH 5.5 to pH 5.0	BITI ITI-E7
STRONG	10^{-9} M to 10^{-11} M	0.03% to 0.06%	pH 5.0 to pH4.5	BITI-E7 BITI-E7-1222 AMINO1 AMINO2 MUTP1
VERY STRONG	$K_D < 10^{-11}$ M	>0.1%	≤ pH 4.0	BITI-E7-141 MUTT26A MUTQE MUT1619

TABLE 213

Fractionation of EpiC-10 and MA-ITI phage on Cat-G beads

	EpiC-10		MA-ITI	
Sample	Total pfu in sample	Fraction of input	Total pfu in sample	Fraction of input
INPUT	$5.0 \cdot 10^{11}$	1.00	$4.6 \cdot 10^{11}$	1.00
Final TBS-TWEEN Wash	$1.8 \cdot 10^{7}$	$3.6 \cdot 10^{-5}$	$7.1 \cdot 10^{6}$	$1.5 \cdot 10^{-5}$
pH 7.0	$1.5 \cdot 10^{7}$	$3.0 \cdot 10^{-5}$	$6.1 \cdot 10^{6}$	$1.3 \cdot 10^{-5}$
pH 6.0	$2.3 \cdot 10^{7}$	$4.6 \cdot 10^{-5}$	$2.3 \cdot 10^{6}$	$5.0 \cdot 10^{-6}$
pH 5.5	$2.5 \cdot 10^{7}$	$5.0 \cdot 10^{-5}$	$1.2 \cdot 10^{6}$	$2.6 \cdot 10^{-6}$
pH 5.0	$2.1 \cdot 10^{7}$	$4.2 \cdot 10^{-5}$	$1.1 \cdot 10^{6}$	$2.4 \cdot 10^{-6}$
pH 4.5	$1.1 \cdot 10^{7}$	$2.2 \cdot 10^{-5}$	$6.7 \cdot 10^{5}$	$1.5 \cdot 10^{-6}$
pH 4.0	$1.9 \cdot 10^{6}$	$3.8 \cdot 10^{-6}$	$4.4 \cdot 10^{5}$	$9.6 \cdot 10^{-7}$
pH 3.5	$1.1 \cdot 10^{6}$	$2.2 \cdot 10^{-6}$	$4.4 \cdot 10^{5}$	$9.6 \cdot 10^{-7}$
pH 3.0	$4.8 \cdot 10^{5}$	$9.6 \cdot 10^{-7}$	$3.6 \cdot 10^{5}$	$7.8 \cdot 10^{-7}$
pH 2.5	$2.0 \cdot 10^{5}$	$4.0 \cdot 10^{-7}$	$2.7 \cdot 10^{5}$	$5.9 \cdot 10^{-7}$
pH 2.0	$2.4 \cdot 10^{5}$	$4.8 \cdot 10^{-7}$	$3.2 \cdot 10^{5}$	$7.0 \cdot 10^{-7}$
SUM*	$9.9 \cdot 10^{7}$	$2 \cdot 10^{-4}$	$1.4 \cdot 10^{7}$	$3 \cdot 10^{-5}$

* SUM is the total pfu (or fraction of input) obtained from all pH elution fractions

TABLE 214

Abbreviated fractionation of display phage on hNE beads

	DISPLAY PHAGE			
	EpiNE-7	MA-ITI 2	MA-ITI-E7 1	MA-ITI-E7 2
INPUT (pfu)	1.00 ($1.8 \cdot 10^9$)	1.00 ($1.2 \cdot 10^{10}$)	1.00 ($3.3 \cdot 10^9$)	1.00 ($1.1 \cdot 10^9$)
WASH	$6 \cdot 10^{-5}$	$1 \cdot 10^{-5}$	$2 \cdot 10^{-5}$	$2 \cdot 10^{-5}$
pH 7.0	$3 \cdot 10^{-4}$	$1 \cdot 10^{-5}$	$2 \cdot 10^{-5}$	$4 \cdot 10^{-5}$
pH 3.5	$3 \cdot 10^{-3}$	$3 \cdot 10^{-6}$	$8 \cdot 10^{-5}$	$8 \cdot 10^{-5}$
pH 2.0	$1 \cdot 10^{-3}$	$1 \cdot 10^{-6}$	$6 \cdot 10^{-6}$	$2 \cdot 10^{-5}$
SUM*	$4.3 \cdot 10^{-3}$	$1.4 \cdot 10^{-5}$	$1.1 \cdot 10^{-4}$	$1.4 \cdot 10^{-4}$

* SUM is the total fraction of input pfu obtained from all pH elution fractions

TABLE 211

Effects of antis ra on phage infectivity

Phage (dilution of stock)	Incubation Conditions	pfu/ml	Relative Titer
MA-ITI (10^{-1})	PBS	$1.2 \cdot 10^{11}$	1.00
	NRS	$6.8 \cdot 10^{10}$	0.57
	anti-ITI	$1.1 \cdot 10^{10}$	0.09
MA-ITI (10^{-3})	PBS	$7.7 \cdot 10^{8}$	1.00
	NRS	$6.7 \cdot 10^{8}$	0.87
	anti-ITI	$8.0 \cdot 10^{6}$	0.01
MA (10^{-1})	PBS	$1.3 \cdot 10^{12}$	1.00
	NRS	$1.4 \cdot 10^{12}$	1.10
	anti-ITI	$1.6 \cdot 10^{12}$	1.20
MA (10^{-3})	PBS	$1.3 \cdot 10^{10}$	1.00
	NRS	$1.2 \cdot 10^{10}$	0.92
	anti-ITI	$1.5 \cdot 10^{10}$	1.20

TABLE 212

Fractionation of EpiNE-7 and MA-ITI phage on hNE beads

Sample	EpiNE-7		MA-ITI	
	Total pfu in sample	Fraction of input	Total pfu in sample	Fraction of input
INPUT	$3.3 \cdot 10^{9}$	1.00	$3.4 \cdot 10^{11}$	1.00
Final TBS-TWEEN Wash	$3.8 \cdot 10^{5}$	$1.2 \cdot 10^{-4}$	$1.8 \cdot 10^{6}$	$5.3 \cdot 10^{-6}$
pH 7.0	$6.2 \cdot 10^{5}$	$1.8 \cdot 10^{-4}$	$1.6 \cdot 10^{6}$	$4.7 \cdot 10^{-6}$
pH 6.0	$1.4 \cdot 10^{6}$	$4.1 \cdot 10^{-4}$	$1.0 \cdot 10^{6}$	$2.9 \cdot 10^{-6}$
pH 5.5	$9.4 \cdot 10^{5}$	$2.8 \cdot 10^{-4}$	$1.6 \cdot 10^{6}$	$4.7 \cdot 10^{-6}$
pH 5.0	$9.5 \cdot 10^{5}$	$2.9 \cdot 10^{-4}$	$3.1 \cdot 10^{5}$	$9.1 \cdot 10^{-7}$
pH 4.5	$1.2 \cdot 10^{6}$	$3.5 \cdot 10^{-4}$	$1.2 \cdot 10^{5}$	$3.5 \cdot 10^{-7}$
pH 4.0	$1.6 \cdot 10^{6}$	$4.8 \cdot 10^{-4}$	$7.2 \cdot 10^{4}$	$2.1 \cdot 10^{-7}$
pH 3.5	$9.5 \cdot 10^{5}$	$2.9 \cdot 10^{-4}$	$4.9 \cdot 10^{4}$	$1.4 \cdot 10^{-7}$
pH 3.0	$6.6 \cdot 10^{5}$	$2.0 \cdot 10^{-4}$	$2.9 \cdot 10^{4}$	$8.5 \cdot 10^{-8}$
pH 2.5	$1.6 \cdot 10^{5}$	$4.8 \cdot 10^{-5}$	$1.4 \cdot 10^{4}$	$4.1 \cdot 10^{-8}$
pH 2.0	$3.0 \cdot 10^{5}$	$9.1 \cdot 10^{-5}$	$1.7 \cdot 10^{4}$	$5.0 \cdot 10^{-8}$
SUM*	$6.4 \cdot 10^{6}$	$3 \cdot 10^{-3}$	$5.7 \cdot 10^{6}$	$2 \cdot 10^{-5}$

* SUM is the total pfu (or fraction of input) obtained from all pH elution fractions

TABLE 215

Fractionation of EpiNE-7 and MA-ITI-E7 phage on hNE beads

Sample	EpiNE-7		MA-ITI-E7	
	Total pfu in sample	Fraction of input	Total pfu in sample	Fraction of input
INPUT	$1.8 \cdot 10^{9}$	1.00	$3.0 \cdot 10^{9}$	1.00
pH 7.0	$5.2 \cdot 10^{5}$	$2.9 \cdot 10^{-4}$	$6.4 \cdot 10^{4}$	$2.1 \cdot 10^{-5}$
pH 6.0	$6.4 \cdot 10^{5}$	$3.6 \cdot 10^{-4}$	$4.5 \cdot 10^{4}$	$1.5 \cdot 10^{-5}$
pH 5.5	$7.8 \cdot 10^{5}$	$4.3 \cdot 10^{-4}$	$5.0 \cdot 10^{4}$	$1.7 \cdot 10^{-5}$
pH 5.0	$8.4 \cdot 10^{5}$	$4.7 \cdot 10^{-4}$	$5.2 \cdot 10^{4}$	$1.7 \cdot 10^{-5}$
pH 4.5	$1.1 \cdot 10^{6}$	$6.1 \cdot 10^{-4}$	$4.4 \cdot 10^{4}$	$1.5 \cdot 10^{-5}$
pH 4.0	$1.7 \cdot 10^{6}$	$9.4 \cdot 10^{-4}$	$2.6 \cdot 10^{4}$	$8.7 \cdot 10^{-6}$
pH 3.5	$1.1 \cdot 10^{6}$	$6.1 \cdot 10^{-4}$	$1.3 \cdot 10^{4}$	$4.3 \cdot 10^{-6}$
pH 3.0	$3.8 \cdot 10^{5}$	$2.1 \cdot 10^{-4}$	$5.6 \cdot 10^{3}$	$1.9 \cdot 10^{-6}$
pH 2.5	$2.8 \cdot 10^{5}$	$1.6 \cdot 10^{-4}$	$4.9 \cdot 10^{3}$	$1.6 \cdot 10^{-6}$
pH 2.0	$2.9 \cdot 10^{5}$	$1.6 \cdot 10^{-4}$	$2.2 \cdot 10^{3}$	$7.3 \cdot 10^{-7}$
SUM*	$7.6 \cdot 10^{6}$	$4.1 \cdot 10^{-3}$	$3.1 \cdot 10^{5}$	$1.1 \cdot 10^{-4}$

* SUM is the total pfu (or fraction of input) obtained from all pH elution fractions

TABLE 216

Fractionation of MA-EpiNE-7, MA-BITI and MA-BITI-E7 on hNE beads

	MA-BITI		MA-BITI-E7	
Sample	Total pfu in sample	Fraction of input	Total pfu in sample	Fraction of input
INPUT	$2.0 \cdot 10^{10}$	1.00	$6.0 \cdot 10^{9}$	1.00
pH 7.0	$2.4 \cdot 10^{5}$	$1.2 \cdot 10^{-5}$	$2.8 \cdot 10^{5}$	$4.7 \cdot 10^{-5}$
pH 6.0	$2.5 \cdot 10^{5}$	$1.2 \cdot 10^{-5}$	$2.8 \cdot 10^{5}$	$4.7 \cdot 10^{-5}$
pH 5.0	$9.6 \cdot 10^{4}$	$4.8 \cdot 10^{-6}$	$3.7 \cdot 10^{5}$	$6.2 \cdot 10^{-5}$
pH 4.5	$4.4 \cdot 10^{4}$	$2.2 \cdot 10^{-6}$	$3.8 \cdot 10^{5}$	$6.3 \cdot 10^{-5}$
pH 4.0	$3.1 \cdot 10^{4}$	$1.6 \cdot 10^{-6}$	$2.4 \cdot 10^{5}$	$4.0 \cdot 10^{-5}$
pH 3.5	$8.6 \cdot 10^{4}$	$4.3 \cdot 10^{-6}$	$9.0 \cdot 10^{4}$	$1.5 \cdot 10^{-5}$
pH 3.0	$2.2 \cdot 10^{4}$	$1.1 \cdot 10^{-6}$	$8.9 \cdot 10^{4}$	$1.5 \cdot 10^{-5}$
pH 2.5	$2.2 \cdot 10^{4}$	$1.1 \cdot 10^{-6}$	$2.3 \cdot 10^{4}$	$3.8 \cdot 10^{-6}$
pH 2.0	$7.7 \cdot 10^{3}$	$3.8 \cdot 10^{-7}$	$8.7 \cdot 10^{3}$	$1.4 \cdot 10^{-6}$
SUM*	$8.0 \cdot 10^{5}$	$3.9 \cdot 10^{-5}$	$1.8 \cdot 10^{6}$	$2.9 \cdot 10^{-4}$

* SUM is the total pfu (or fraction of input) obtained from all pH elution fractions

TABLE 216
(continu d)
Fractionation of MA-EpiNE-7, MA-BITI and MA-BITI-E7 on hNE beads

	MA-EpiNE-7	
Sample	Total pfu in sample	Fraction of input
INPUT	$1.5 \cdot 10^{9}$	1.00
pH 7.0	$2.9 \cdot 10^{5}$	$1.9 \cdot 10^{-4}$
pH 6.0	$3.7 \cdot 10^{5}$	$2.5 \cdot 10^{-4}$
pH 5.0	$4.9 \cdot 10^{5}$	$3.3 \cdot 10^{-4}$
pH 4.5	$6.0 \cdot 10^{5}$	$4.0 \cdot 10^{-4}$
pH 4.0	$6.4 \cdot 10^{5}$	$4.3 \cdot 10^{-4}$
pH 3.5	$5.0 \cdot 10^{5}$	$3.3 \cdot 10^{-4}$
pH 3.0	$1.9 \cdot 10^{5}$	$1.3 \cdot 10^{-4}$
pH 2.5	$7.7 \cdot 10^{4}$	$5.1 \cdot 10^{-5}$
pH 2.0	$9.7 \cdot 10^{4}$	$6.5 \cdot 10^{-5}$
SUM*	$3.3 \cdot 10^{6}$	$2.2 \cdot 10^{-3}$

* SUM is the total pfu (or fraction of input) obtained from all pH elution fractions

TABLE 217

Fractionation of MA-BITI-E7 and MA-BITI-E7-1222 on hNE beads

	MA-BITI-E7		MA-BITI-E7-1222	
Sample	Total pfu in sample	Fraction of input	Total pfu in sample	Fraction of input
INPUT	$1.3 \cdot 10^{9}$	1.00	$1.2 \cdot 10^{9}$	1.00
pH 7.0	$4.7 \cdot 10^{4}$	$3.6 \cdot 10^{-5}$	$4.0 \cdot 10^{4}$	$3.3 \cdot 10^{-5}$
pH 6.0	$5.3 \cdot 10^{4}$	$4.1 \cdot 10^{-5}$	$5.5 \cdot 10^{4}$	$4.6 \cdot 10^{-5}$
pH 5.5	$7.1 \cdot 10^{4}$	$5.5 \cdot 10^{-5}$	$5.4 \cdot 10^{4}$	$4.5 \cdot 10^{-5}$
pH 5.0	$9.0 \cdot 10^{4}$	$6.9 \cdot 10^{-5}$	$6.7 \cdot 10^{4}$	$5.6 \cdot 10^{-5}$
pH 4.5	$6.2 \cdot 10^{4}$	$4.8 \cdot 10^{-5}$	$6.7 \cdot 10^{4}$	$5.6 \cdot 10^{-5}$
pH 4.0	$3.4 \cdot 10^{4}$	$2.6 \cdot 10^{-5}$	$2.7 \cdot 10^{4}$	$2.2 \cdot 10^{-5}$
pH 3.5	$1.8 \cdot 10^{4}$	$1.4 \cdot 10^{-5}$	$2.3 \cdot 10^{4}$	$1.9 \cdot 10^{-5}$
pH 3.0	$2.5 \cdot 10^{3}$	$1.9 \cdot 10^{-6}$	$6.3 \cdot 10^{3}$	$5.2 \cdot 10^{-6}$
pH 2.5	$<1.3 \cdot 10^{3}$	$<1.0 \cdot 10^{-6}$	$<1.3 \cdot 10^{3}$	$<1.0 \cdot 10^{-6}$
pH 2.0	$1.3 \cdot 10^{3}$	$1.0 \cdot 10^{-6}$	$1.3 \cdot 10^{3}$	$1.0 \cdot 10^{-6}$
SUM*	$3.8 \cdot 10^{5}$	$2.9 \cdot 10^{-4}$	$3.4 \cdot 10^{5}$	$2.8 \cdot 10^{-4}$

* SUM is the total pfu (or fraction of input) obtained from all pH elution fractions

TABLE 218

Fractionation of MA-EpiNE7 and MA-BITI-E7-141 on hNE beads

	MA-EpiNE7		MA-BITI-E7-141	
Sample	Total pfu in sample	Fraction of input	Total pfu in sample	Fraction of input
INPUT	$6.1 \cdot 10^{8}$	1.00	$2.0 \cdot 10^{9}$	1.00
pH 7.0	$5.3 \cdot 10^{4}$	$8.7 \cdot 10^{-5}$	$4.5 \cdot 10^{5}$	$2.2 \cdot 10^{-4}$
pH 6.0	$9.7 \cdot 10^{4}$	$1.6 \cdot 10^{-4}$	$4.4 \cdot 10^{5}$	$2.2 \cdot 10^{-4}$
pH 5.5	$1.1 \cdot 10^{5}$	$1.8 \cdot 10^{-4}$	$4.4 \cdot 10^{5}$	$2.2 \cdot 10^{-4}$
pH 5.0	$1.4 \cdot 10^{5}$	$2.3 \cdot 10^{-4}$	$7.2 \cdot 10^{5}$	$3.6 \cdot 10^{-4}$
pH 4.5	$1.0 \cdot 10^{5}$	$1.6 \cdot 10^{-4}$	$1.3 \cdot 10^{6}$	$6.5 \cdot 10^{-4}$
pH 4.0	$2.0 \cdot 10^{5}$	$3.3 \cdot 10^{-4}$	$1.1 \cdot 10^{6}$	$5.5 \cdot 10^{-4}$
pH 3.5	$9.7 \cdot 10^{4}$	$1.6 \cdot 10^{-4}$	$5.9 \cdot 10^{5}$	$3.0 \cdot 10^{-4}$
pH 3.0	$3.8 \cdot 10^{4}$	$6.2 \cdot 10^{-5}$	$2.3 \cdot 10^{5}$	$1.2 \cdot 10^{-4}$
pH 2.5	$1.3 \cdot 10^{4}$	$2.1 \cdot 10^{-5}$	$1.2 \cdot 10^{5}$	$6.0 \cdot 10^{-5}$
pH 2.0	$1.6 \cdot 10^{4}$	$2.6 \cdot 10^{-5}$	$1.0 \cdot 10^{5}$	$5.0 \cdot 10^{-5}$
SUM*	$8.6 \cdot 10^{5}$	$1.4 \cdot 10^{-3}$	$5.5 \cdot 10^{6}$	$2.8 \cdot 10^{-3}$

* SUM is the total pfu (or fraction of input) obtained from all pH elution fractions

TABLE 219

pH Elution Analysis of hNE Bilnding
by BITI-E7-141 Varient Display Phage

DISPLAYED PROTEIN	INPUT PFU[c]	FRACTION OF INPUT RECOVERED AT pH: 7.0[d]	3.5[d]	2.0[d]	RECOVERY TOTAL[e]	RELATIVE[f]
AMINO1[b]	0.96	0.24	2.3	0.35	2.9	0.11
AMINO2[a]	6.1	0.57	2.1	0.45	3.1	0.12
BITI-E7-1222[b]	1.2	0.72	4.0	0.64	5.4	0.21
EpiNE7[b]	0.72	0.44	6.4	2.2	9.0	0.35
MUTP1[a]	3.9	1.8	9.2	1.2	12	0.46
MUT1619[b]	0.78	0.82	9.9	0.84	12	0.46
MUTQE[a]	4.7	1.2	16	5.3	22	0.85
MUTT26A[b]	0.51	2.5	19	3.3	25	0.96
BITI-E7-141[a]	1.7	2.2	18	5.4	26	1.00
BITI-E7-141[b]	0.75	2.1	21	3.2	26	1.00

[a] results from abbreviated pH elution protocol
[b] results from extended pH elution protocol
[c] units are 10^9 pfu
[d] units are 10^{-4}
[e] sum of pH 7.0, pH 3.5, and pH 2.0 recoveries, units are 10^{-4}
[f] total fraction of input recovered divided by total fraction of input recovered for BITI-E7-141

TABLE 220

WEAK ($K_D > 10^{-8}$ M)

1. KEDSCQLGYSAGPCMGMTSRYFYNGTSMACETFQYGGCMGNGNNFVTEKDCLQTCRGA

MODERATE ($10^{-8} > K_D > 10^{-9}$)

2. KEDSCQLGYSAGPC**V**A**M**F**P**RYFYNGTSMACETFQYGGCMGNGNNFVTEKDCLQTCRGA
3. **R**P**D**FCQLGYSAGPCMGMTSRYFYNGTSMACETFQYGGCMGNGNNFVTEKDCLQTCRGA

STRONG ($10^{-9} > K_D > 10^{-11}$D)

4. **R**P**D**F**C**QLGYSAGPC**V**A**M**F**P**RYFYNGTSMACETFQYGGCMGNGNNFVTEKDCLQTCRGA
5. **R**P**D**F**C**QLGYS**T**GPC**V**A**M**F**P**RYFYNGTSMACETFQYGGCMGNGNNFVTEKDCLQTCRGA
6. KED**F**CQLGYSAGPC**V**A**M**F**P**RYFYNGTSMACETFQYGGCMGNGNNFVTEKDCLQTCRGA
7. K**P**DSCQLGYSAGPC**V**A**M**F**P**RYFYNGTSMACETFQYGGCMGNGNNFVTEKDCLQTCRGA
8. **R**P**D**F**C**QLGYSAGPC**I**GM**F**SRYFYNGTSMACETFQYGGCMGNGNNFVTEKDCLQTCRGA

VERY STRONG ($K_D < 10^{-11}$ M)

```
                  1111111111222222222233333333334444444444555555555
         123456789012345678901234567890123456789012345678901234567 8
9.       RPDFCQLGYSAGPCVAMFPRYFYNGTSMACQTFVYGGCMGNGNNFVTEKDCLQTCRGA
10.      RPDFCQLGYSAGPCVAMFPRYFYNGASMACQTFVYGGCMGNGNNFVTEKDCLQTCRGA
11.      RPDFCQLGYSAGPCVAMFPRYFYNGTSMACETFVYGGCMGNGNNFVTEKDCLQTCRGA
12.      RPDFCQLGYSAGPCVGMFSRYFYNGTSMACQTFVYGGCMGNGNNFVTEKDCLQTCRGA
```

Residues shown underlined and bold are changed from those present in ITI-D1.

Sequences Key:

1. ITI-D1
2. ITI-E7
3. BITI
4. BITI-E7
5. BITI-E7-1222
6. AMINO1
7. AMINO2
8. MUTP1
9. BITI-E7-141
10. MUTT26A
11. MUTQE
12. MUT1619

TABLE 221
Information same as in Table 220, but focuses on sites where alterations were made

WEAK ($K_D > 10^{-6}$ M)

1. KEDSCQLGYSAGPCMGMTSRYFYNGTSMACETFQYGGCMGNGNNFVTEKDCLQTCRGA
1. KE.S.....A...MGMTS.....T....E..Q.....

MODERATE ($10^{-6} > K_D > 10^{-9}$)

2. KE.S.....A...VAMFP.....T....E..Q.....
3. RP.F.....A...MGMTS.....T....E..Q.....

STRONG ($10^{-9} > K_D > 10^{-11}$D)

4. RP.F.....A...VAMFP.....T....E..Q.....
5. RP.F.....T...VAMFP.....T....E..Q.....
6. KE.F.....A...VAMFP.....T....E..Q.....
7. KP.S.....A...VAMFP.....T....E..Q.....
8. RP.F.....A...IGMFS.....T....E..Q.....

VERY STRONG ($K_D < 10^{-11}$ M)

9. RP.F.....A...VAMFP.....T....Q..V.....
10. RP.F.....A...VAMFP.....A....Q..V.....
11. RP.F.....A...VAMFP.....T....E..V.....
12. RP.F.....A...VGMFS.....T....Q..V.....

Sequence key same as in Table 220

CLAIMS

1. An inhibitor of human neutrophil elastase which is selected from the group consisting of EpiNEα, EpiNE1, EpiNE2, EpiNE3, EpiNE4, EpiNE5, EpiNE6, EpiNE7, EPINE8.

2. An inhibitor of human cathepsin G which is selected from the group consisting of EpiC1, EpiC7, EpiC8, EpiC10, EpiC20, EpiC31, EpiC32, EpiC33, EpiC34, and EpiC35.

3. An inhibitor of human neutrophil elastase which is selected from the group consisting of ITI-E7, BITI-E7, BITI-E7-1222, AMINO1, AMINO2, MUTP1, BITI-E7-141, MUTT26A, MUTQE, and MUT1619 in Table 220.

4. A homologous inhibitor of a reference inhibitor according to claims 1-3, said homologous inhibitor differing from said reference inhibitor by one or more substitutions of class A according to Table 65.

5. A homologous inhibitor of a reference inhibitor according to claims 1-3, said homologous inhibitor differing from said reference inhibitor by one or more substitutions of class A or B according to Table 65.

6. A homologous inhibitor of a reference inhibitor according to claims 1-3, said homologous inhibitor differing from said reference inhibitor by one or more substitutions of class A, B or C according to Table 65.

7. An inhibitor of human neutrophil elastase which is a compound having the formula of Figure 8, wherein

R_1 is hydrogen, L-prolyl, L,L cystinyl (i.e. NH_2-CH(CH_2-S-S-CH_2-CH(NH_2)-COOH)-CO-), L-valyl, other amino acids, or a carboxylic acid derivative having 2-8 carbons,

$R_2$is 2-propyl or secondary butyl,
Xis -CO-CH_2-, -CO-CFH-, -CO-CFH-CH_2-, -CO-CF_2-, -CO-CF_2-CH_2-, -B(OH)-CH_2-, -B(OR_7)-CH_2-, -SO-CH_2-, -CO-S, or -CO-CO-,
$R_3$is -H, -CH_3, -CH_2-COOH, or -CH_2-CH_2-COOH,
R4 is -CH_2-phenyl or -CH_2-CH_2-S-CH_3,
$R_5$is -CH_2-phenyl or other arylmethyl group,
$R_6$is -NH_2, -OH, or an additional N-linked amino acid, and
$R_7$is a small alkyl group.

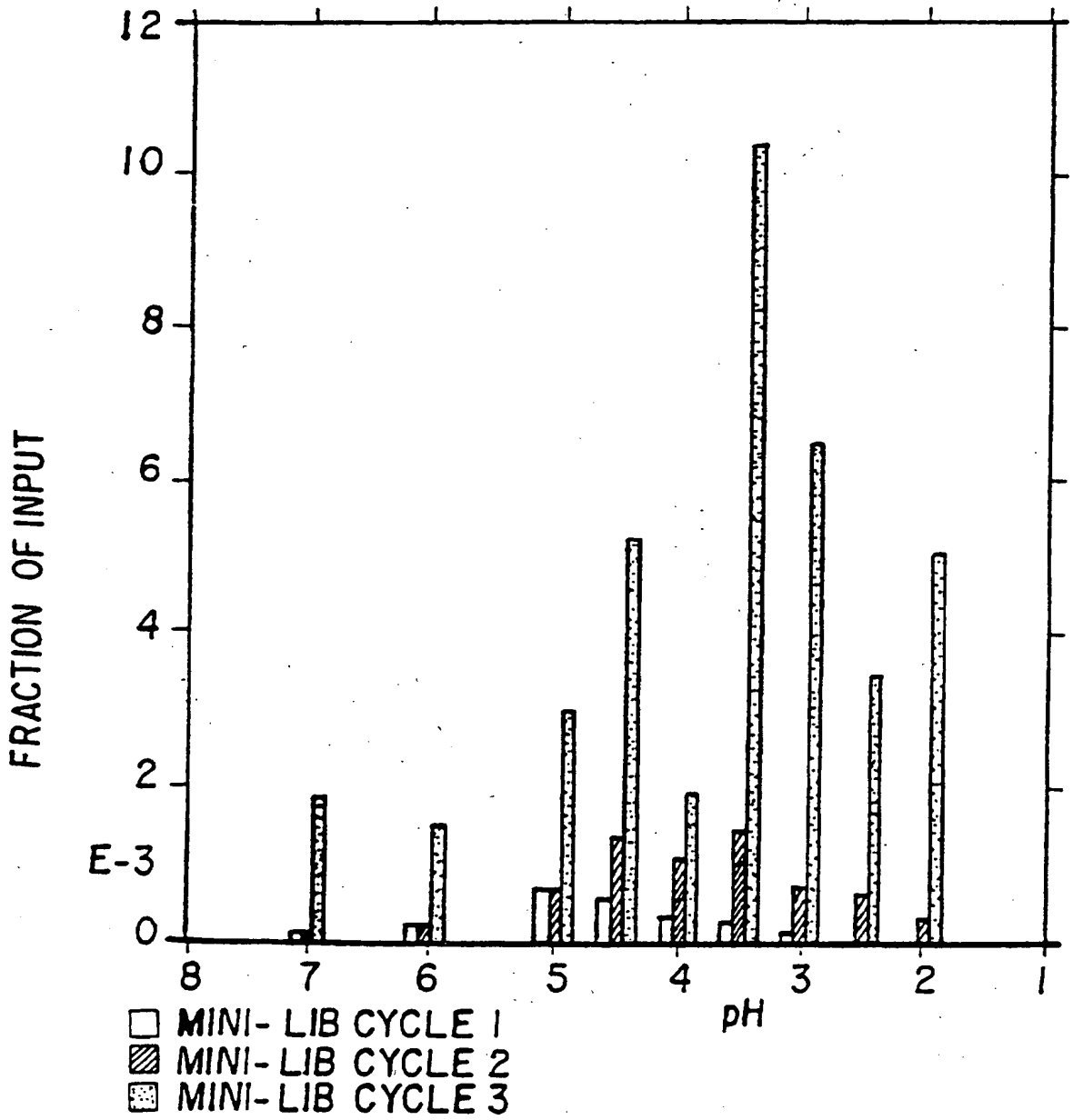
8. An inhibitor of human neutrophil elastase which is a compound having the formula of Figure 9, wherein
R_1 is a relatively rigid bifunctional linker,
R_2 is 2-propyl or secondary butyl,
X is -CO-CH_2-, -CO-CFH-, -CO-CFH-CH_2-, -CO-CF_2-, -CO-CF_2-CH_2-, -B(OH)-CH_2-, -B(OR_7)-CH_2-, -SO-CH_2-, -CO-S, or -CO-CO-,
R_3 is -H, -CH_3, -CH_2-COOH, or -CH_2-CH_2-COOH,
R4 is -CH_2-phenyl or -CH_2-CH_2-S-CH_3,
$R_5$is -CH_2-phenyl or other arylmethyl group,
$R_6$is -NH_2, -OH, or an additional N-linked amino acid, and
$R_7$is a small alkyl group.

9. The inhibitor of claim 8 wherein R_1 is a tricyclic aromatic ring system having diametrically opposed functionalities one of which allows linkage to the amino group attached to C_7 and another that allows linkage to the carbonyl carbon labeled C_{11}.

10. The inhibitor of claim 9 wherein R_1 is 2-carboxymethyl-6-aminomethyl anthraquinone.

11. An inhibitor of human neutrophil elastase which is a compound having the formula of Figure 12, wherein
D1 and D2 are, independently, a hydroxyl group or a group that is capable of being hydrolyzed in aqueous solution to a hydroxyl

FIG. 1.


12
10
8
6
4
2
E-3
0
FRACTION OF INPUT
8
7
6
5
4
3
2
1
pH
MINI-LIB CYCLE 1
MINI-LIB CYCLE 2
MINI-LIB CYCLE 3

group at physiological conditions,

R_3 is -H, $-CH_3$, $-CH_2-COOH$, or $-CH_2-CH_2-COOH$,

R_4 is $-CH_2$-phenyl or $-CH_2-CH_2-S-CH_3$,

R_5 is $-CH_2$-phenyl or other arylmethyl group,

R_6 is $-NH_2$, -OH, or an additional N-linked amino acid.

12. Use of an inhibitor according to any of claims 1-11 in the manufacture of a composition for the treatment or prophylaxis of a condition of the body caused by excessive neutrophil elastase activity.

13. Use of an inhibitor according to any of claims 1-11 in the manufacture of a composition for the treatment or prophylaxis of a condition of the body caused by excessive cathepsin G activity.

FIG. 2.

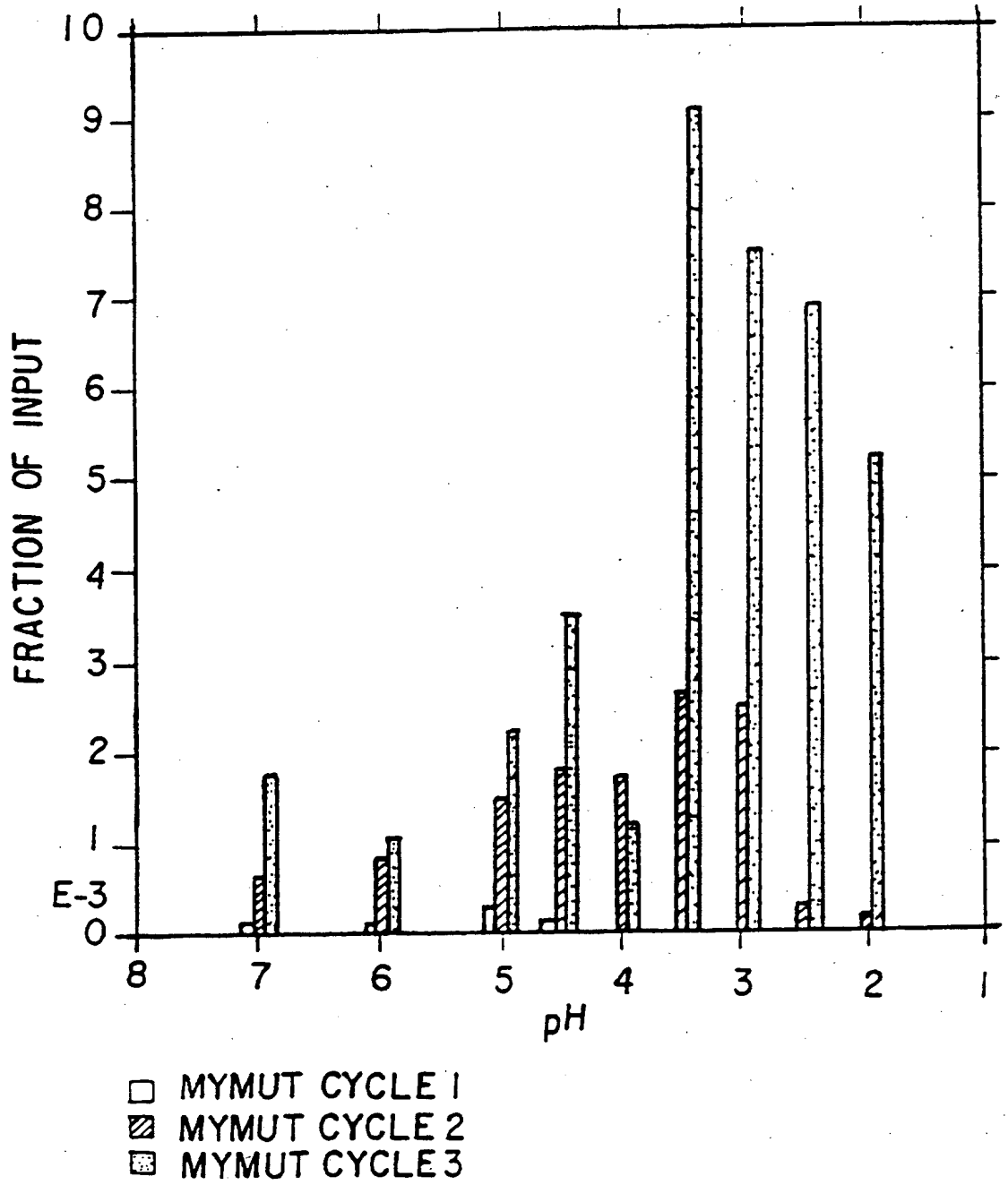

10
9
8
7
6
5
4
3
2
1
E-3
0
FRACTION OF INPUT
8
7
6
5
4
3
2
1
pH
MYMUT CYCLE 1
MYMUT CYCLE 2
MYMUT CYCLE 3

FIG.3.

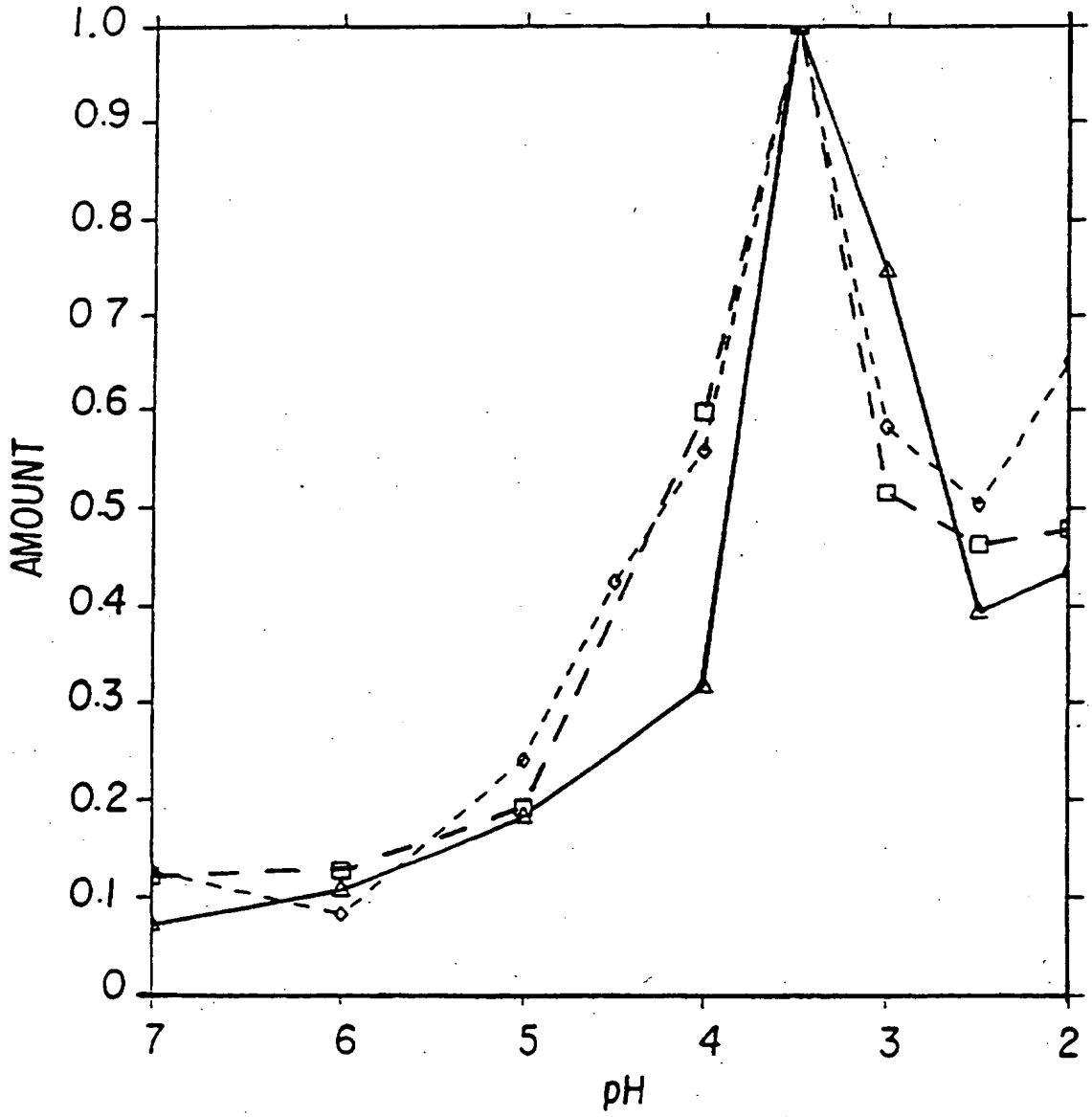

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
AMOUNT
7
6
5
4
3
2
pH
EPINE 1
EPINE 3
EPINE 7

FIG. 4.

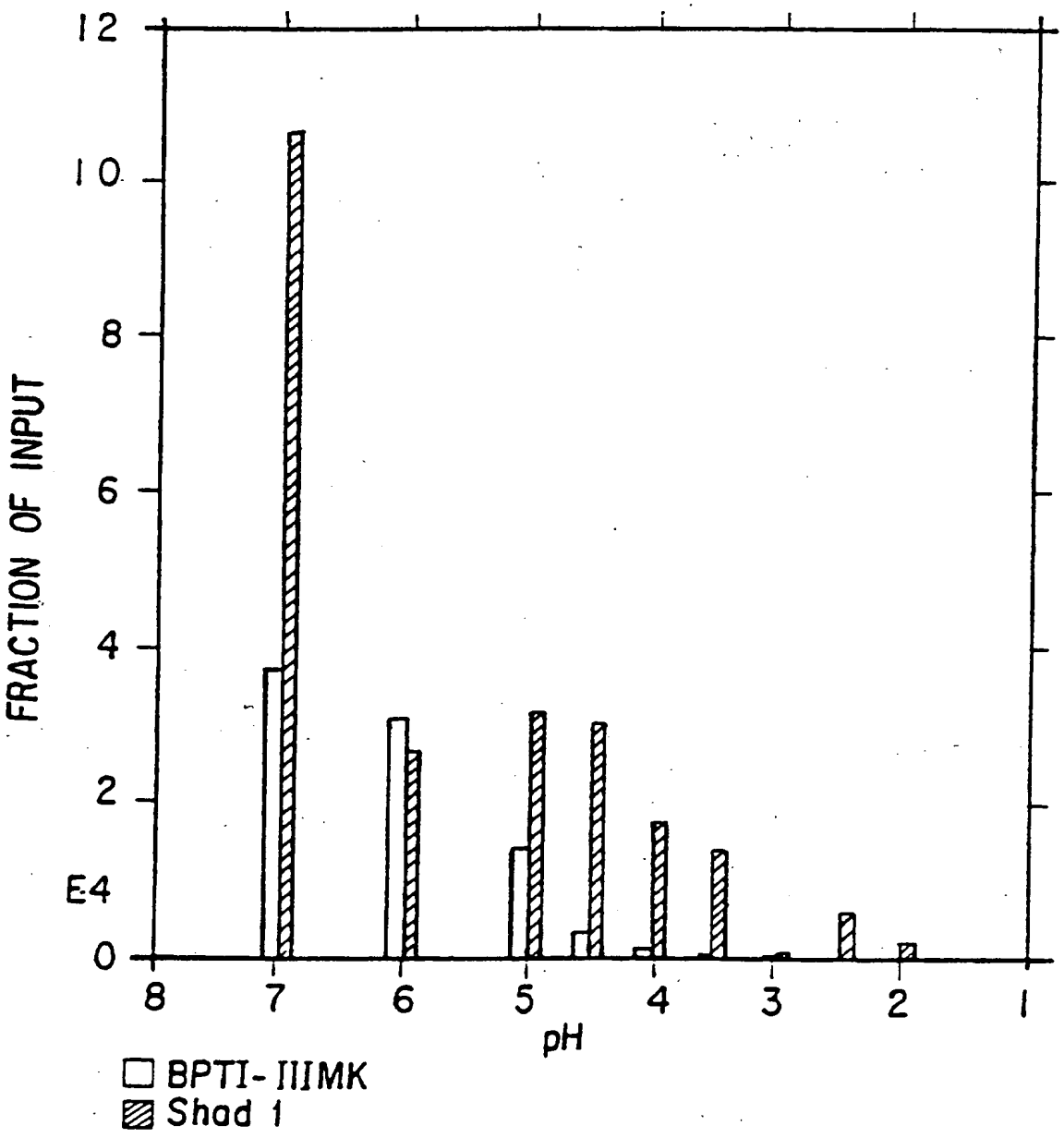

12
10
8
6
4
2
E4
0
FRACTION OF INPUT
8
7
6
5
4
3
2
1
pH
BPTI-IIIMK
Shad 1

FIG. 5.

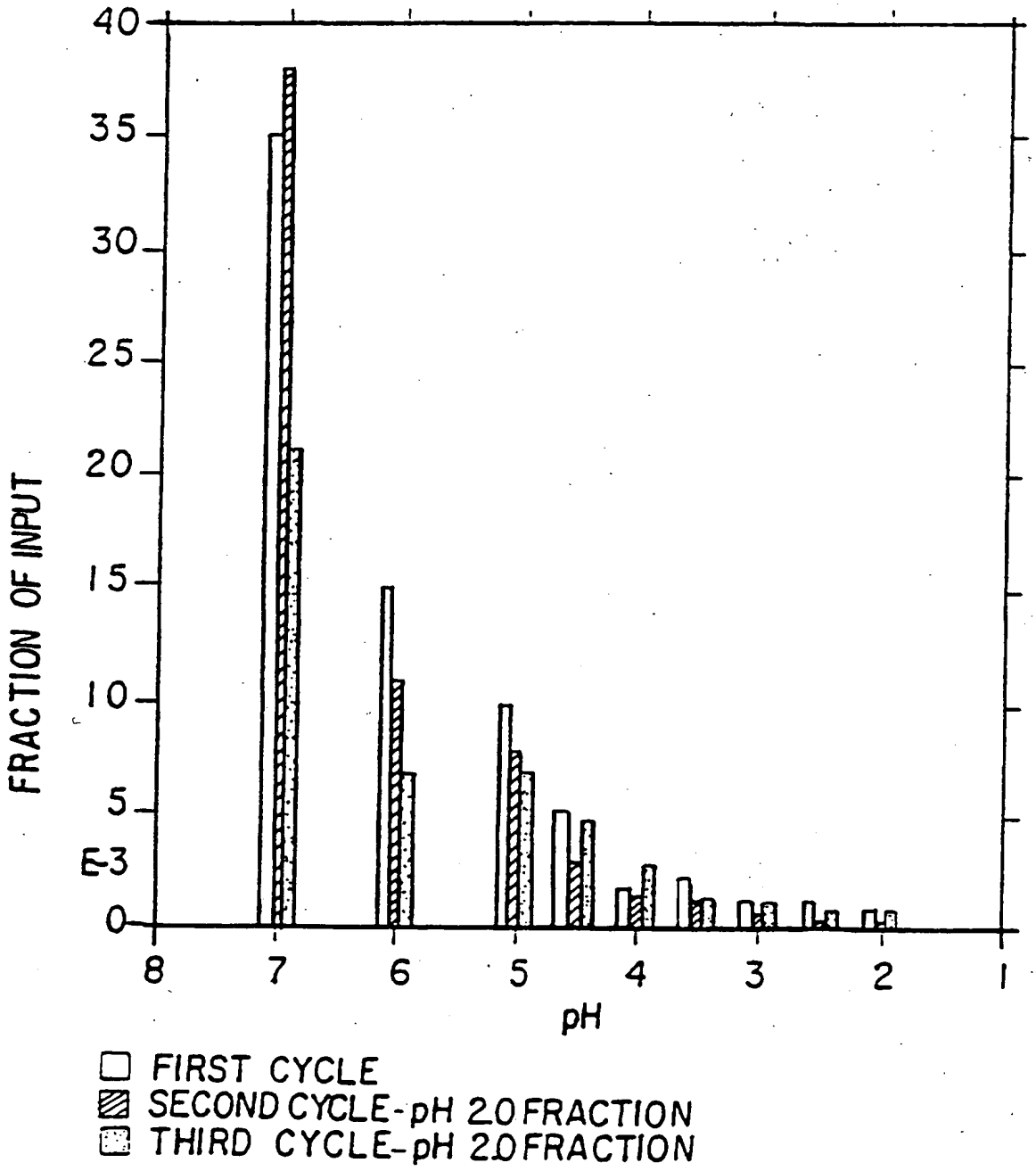

40
35
30
25
20
15
10
5
E-3
0
FRACTION OF INPUT
8
7
6
5
4
3
2
1
pH
FIRST CYCLE
SECOND CYCLE-pH 2.0 FRACTION
THIRD CYCLE-pH 2.0 FRACTION

FIG 6

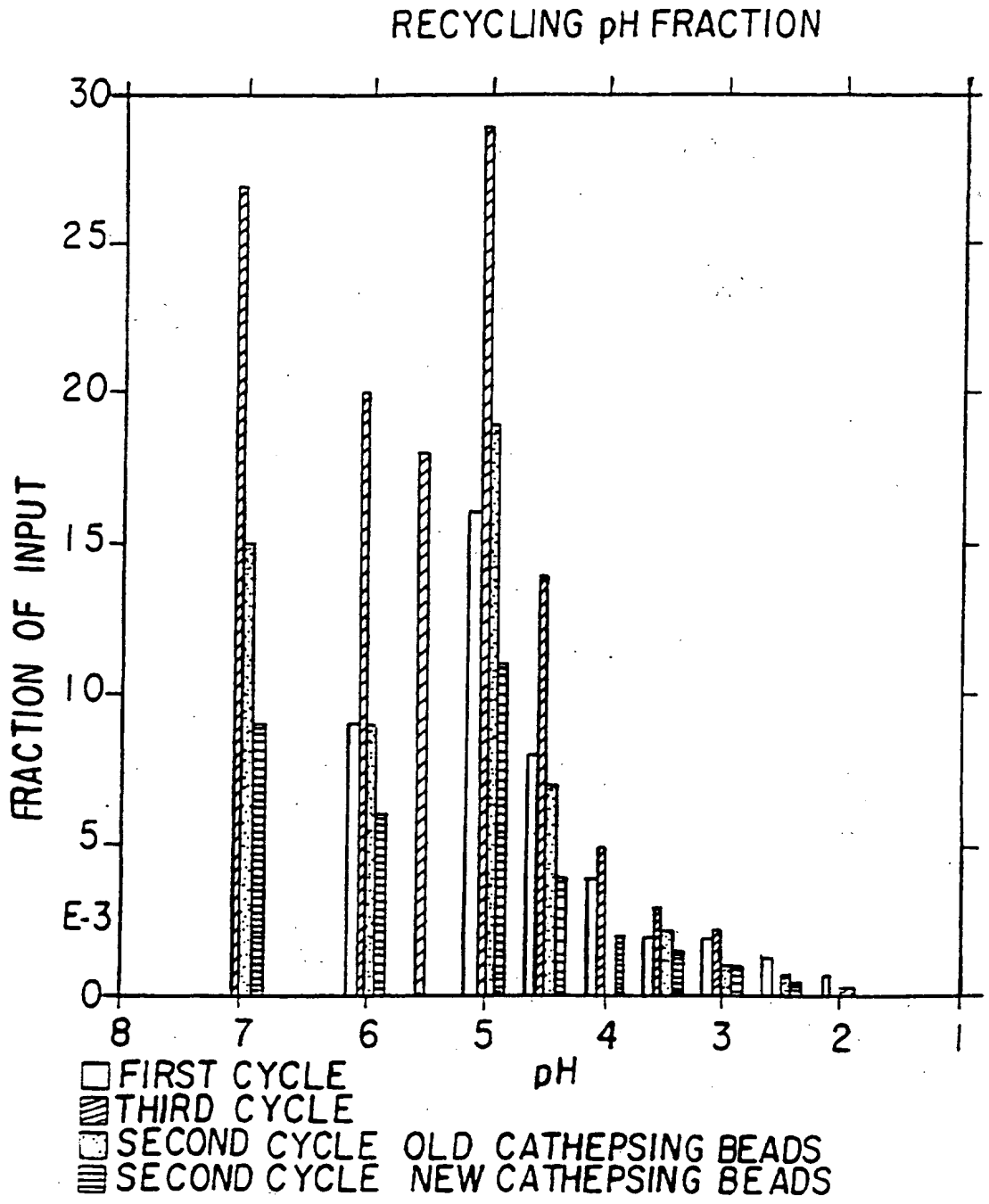

RECYCLING pH FRACTION
FRACTION OF INPUT
30
25
20
15
10
5
E-3
0
8
7
6
5
4
3
2
1
pH
FIRST CYCLE
THIRD CYCLE
SECOND CYCLE OLD CATHEPSING BEADS
SECOND CYCLE NEW CATHEPSING BEADS

FIG.7.

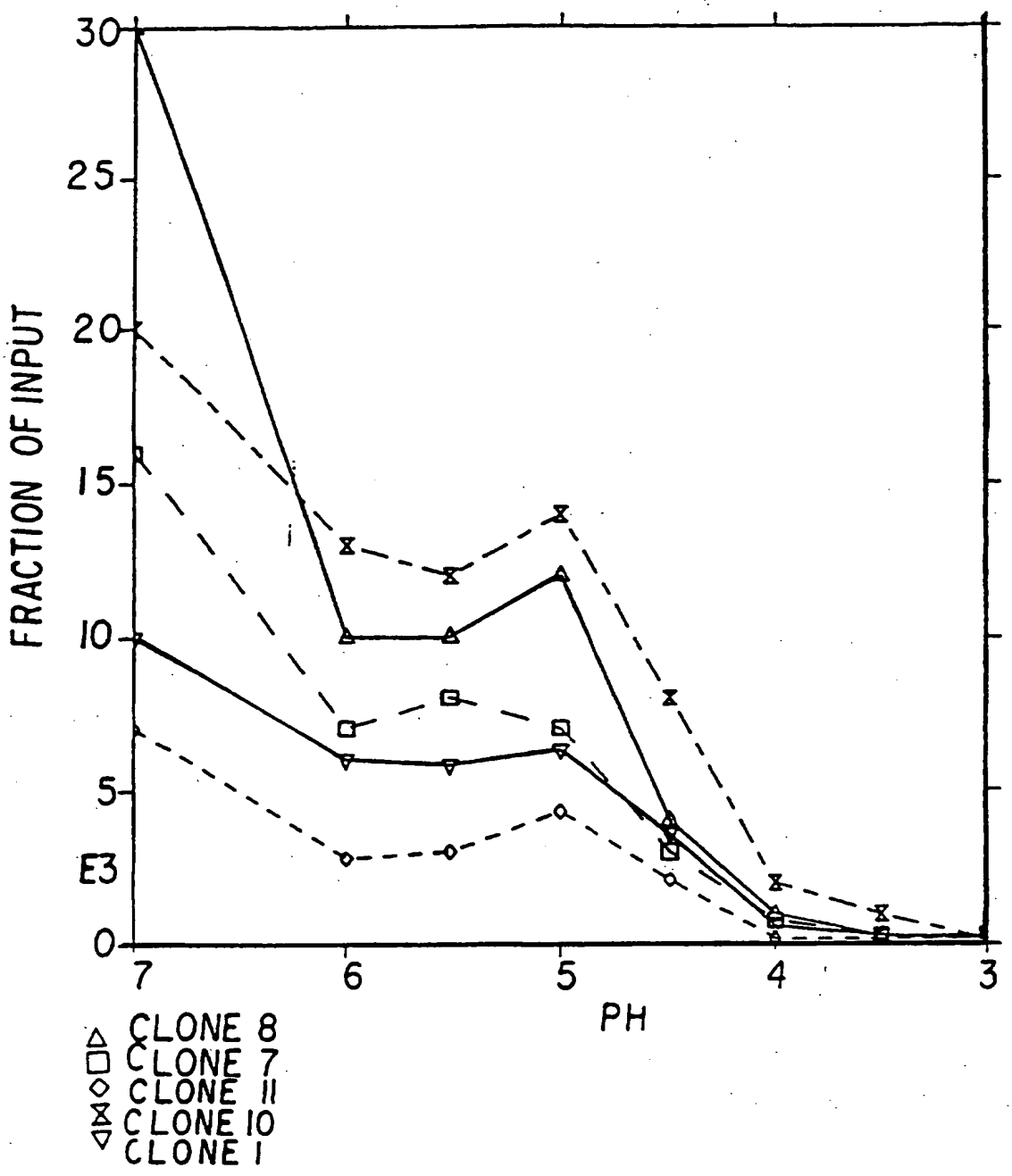
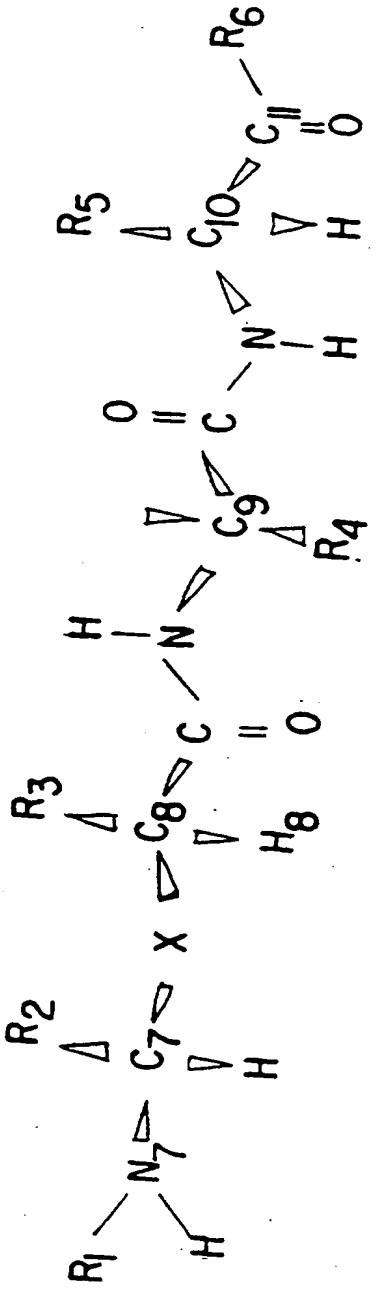

30
25
20
15
10
5
E3
0
FRACTION OF INPUT
7
6
5
4
3
PH
CLONE 8
CLONE 7
CLONE 11
CLONE 10
CLONE 1

FIG. 8.



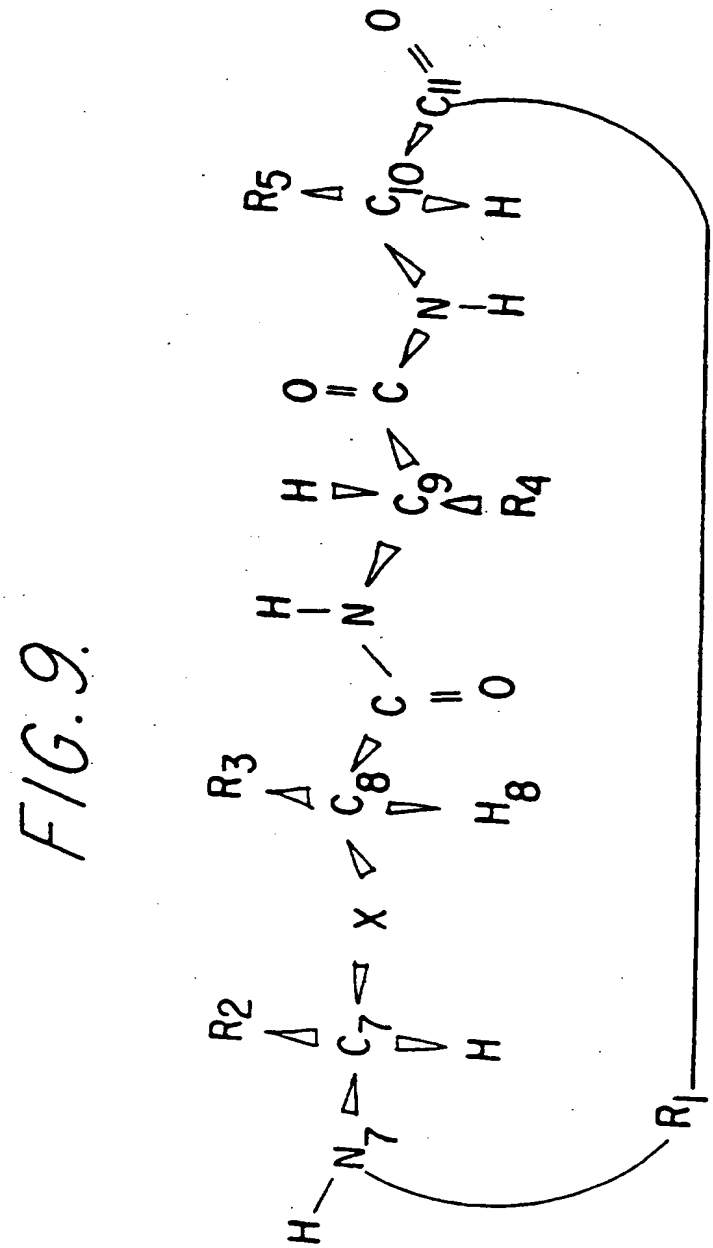


FIG. 9.

FIG. 10.

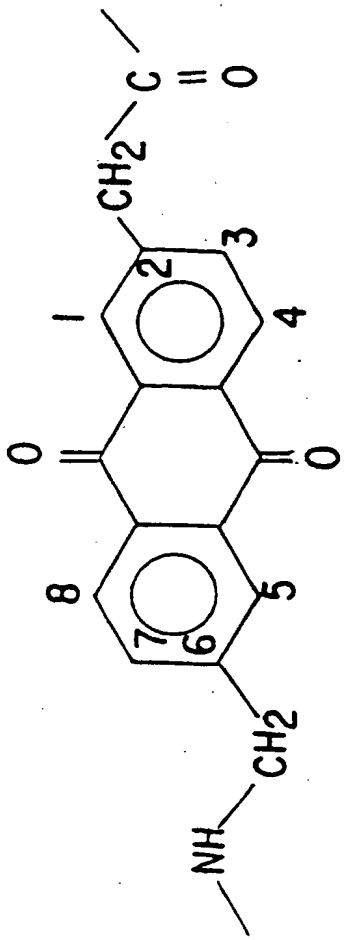
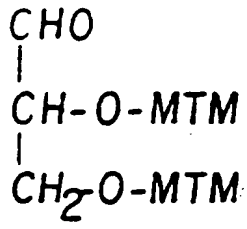
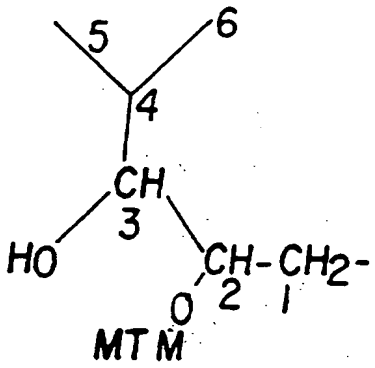


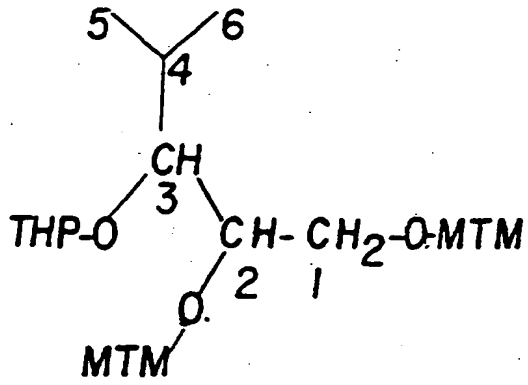
FIG IIA



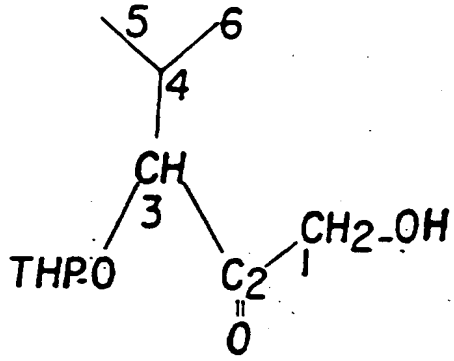
I



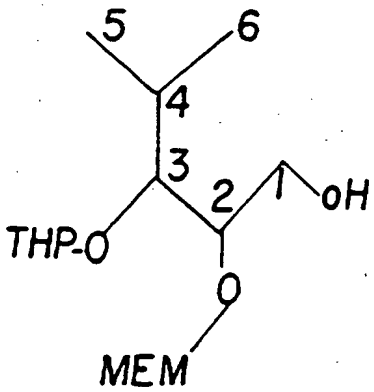
II



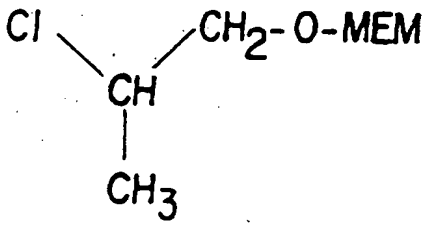
III



IV

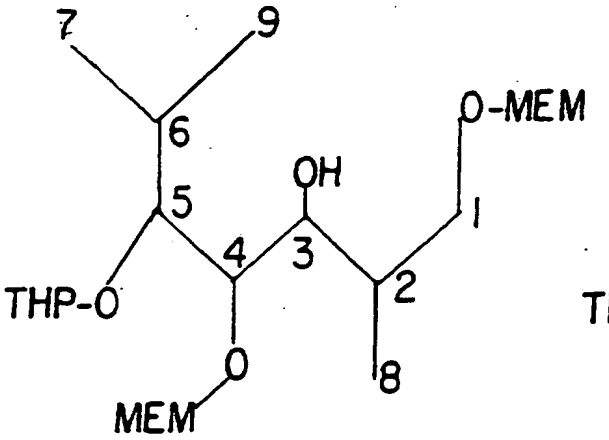


V

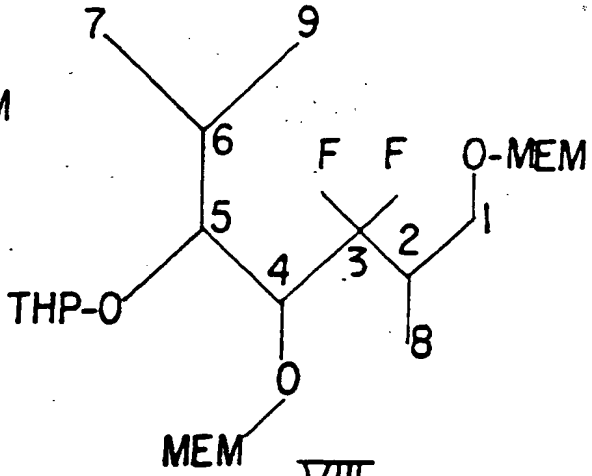


VI

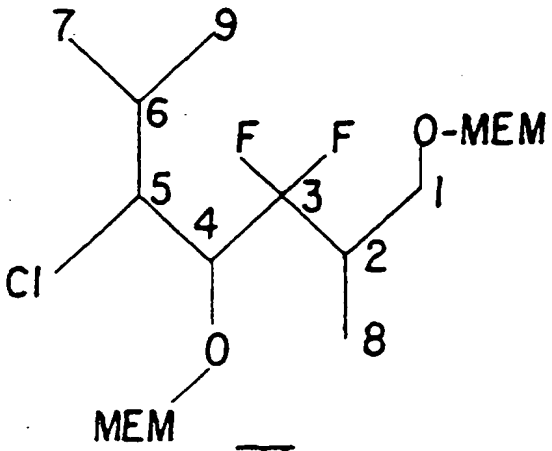
FIG. 11B



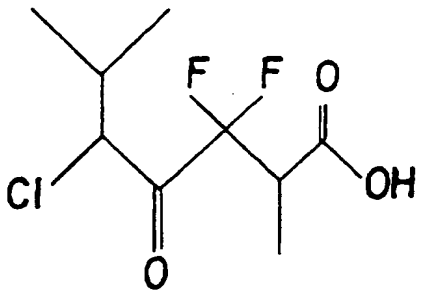
VII



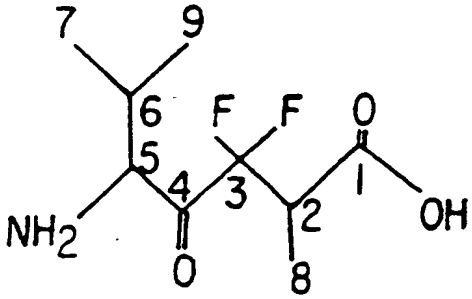
VIII



IX

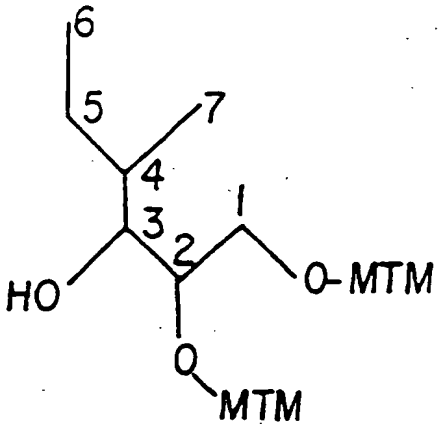


X

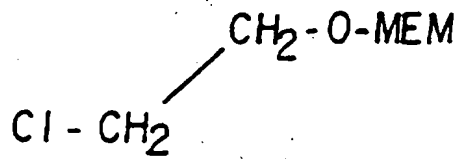


XI

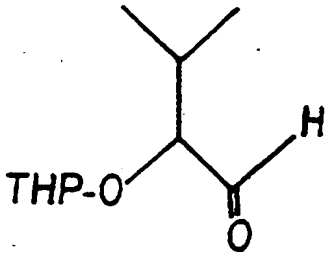
FIG. IIC



XII

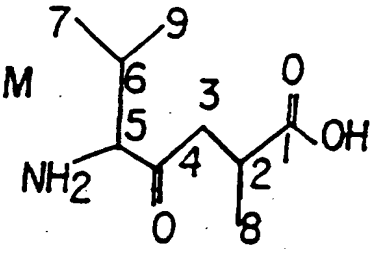


XIII

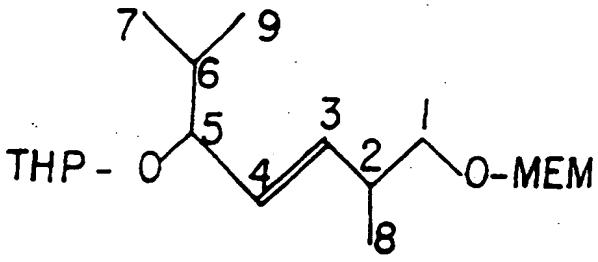


XIV

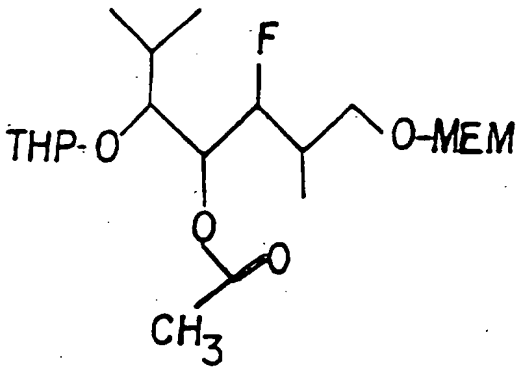
XV



XVI



XVII



XVIII

FIG. 12.

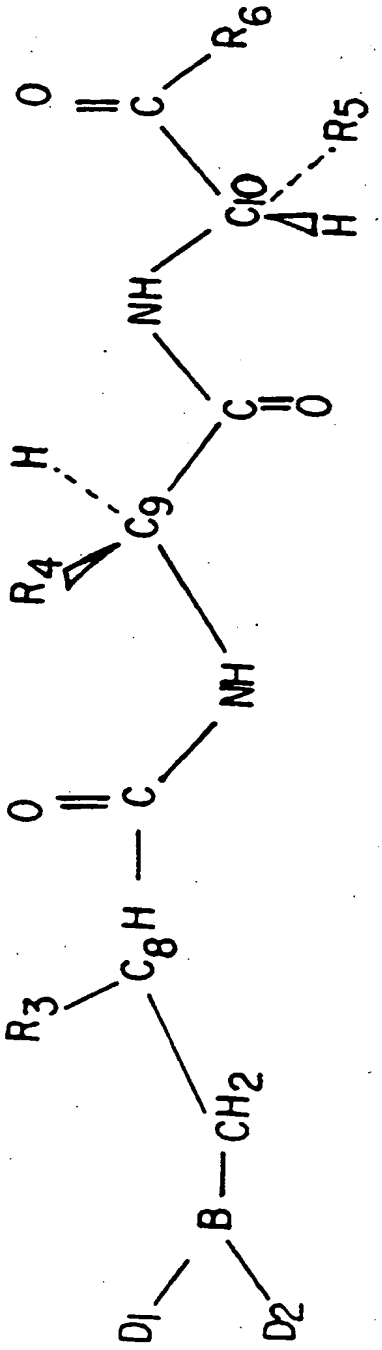
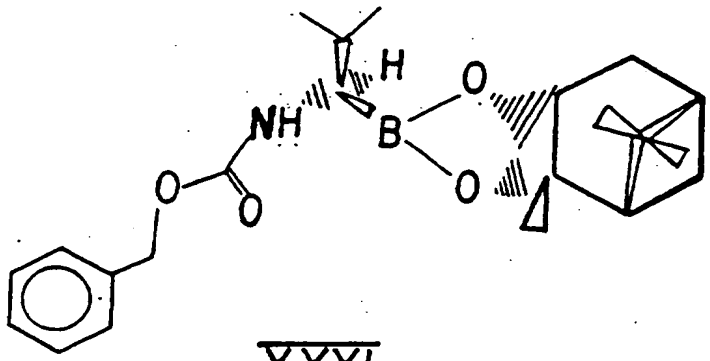
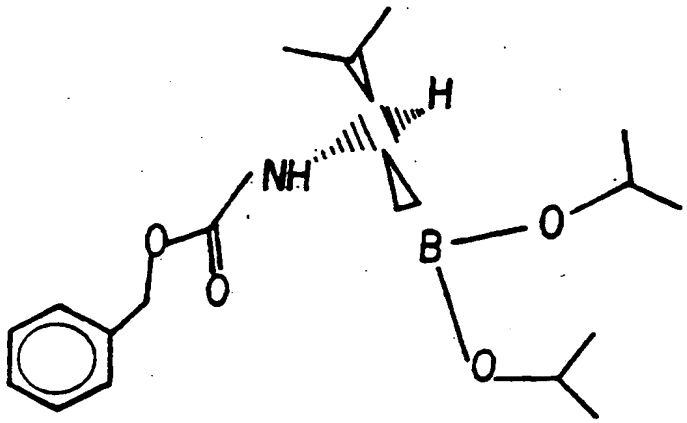


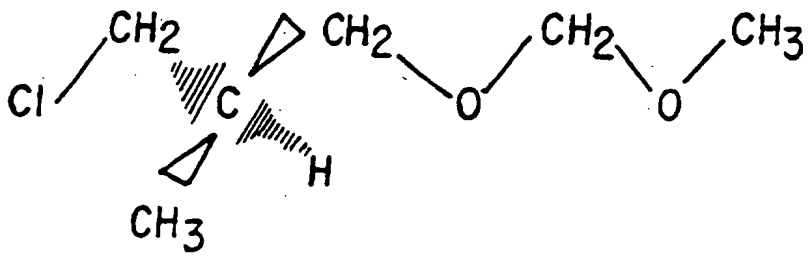
FIG. 13A



XXXI

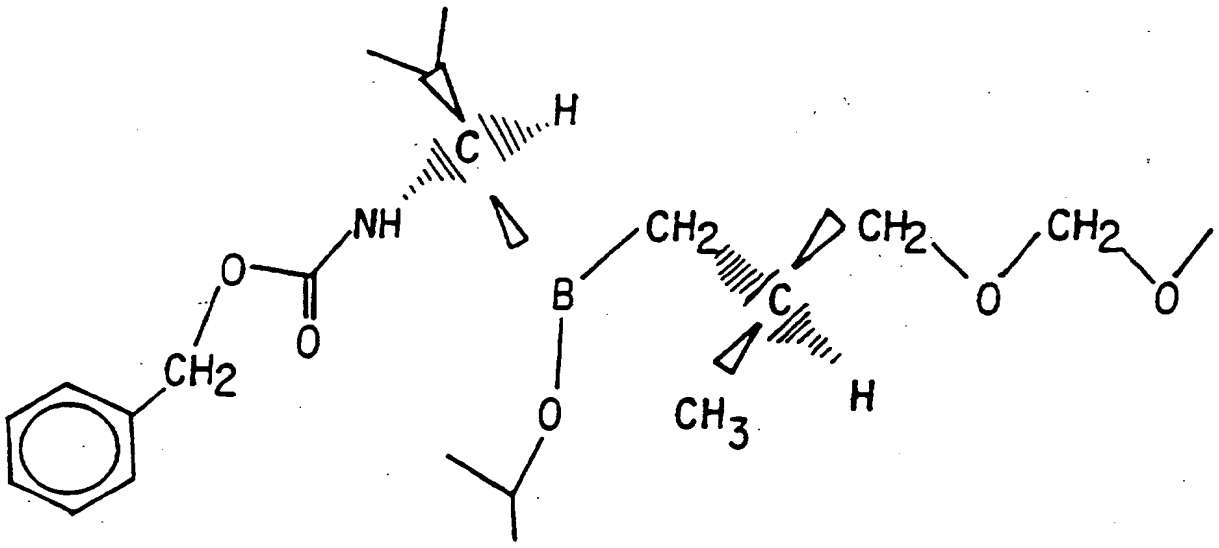


XXXII

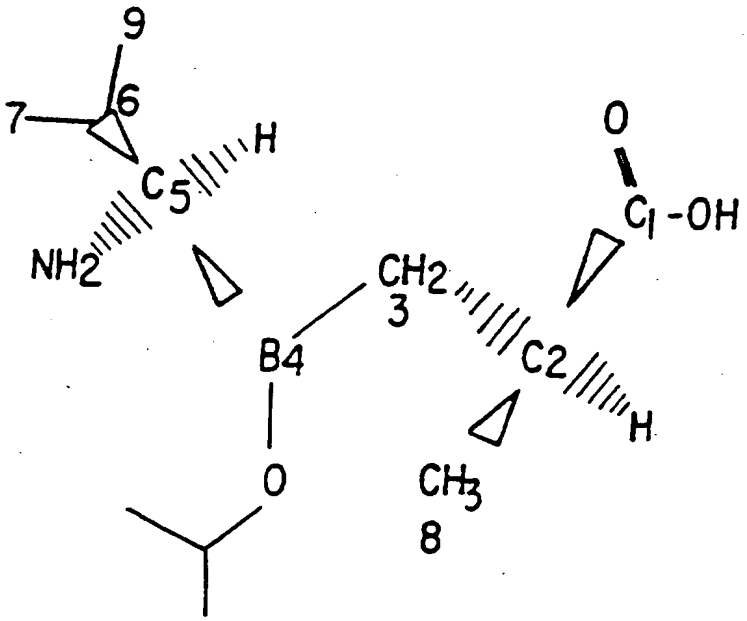


XXXIII

FIG. 13.B

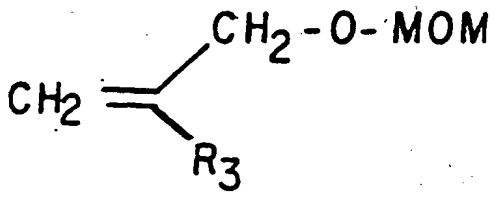


XXXIV

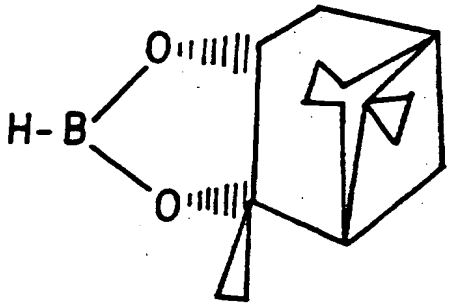


XXXV

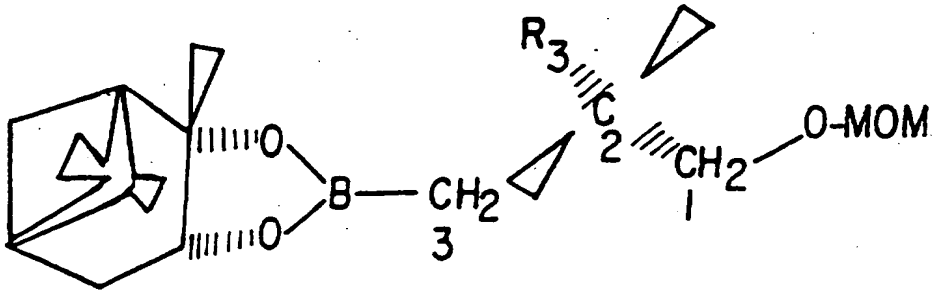
FIG 14 A



XLI

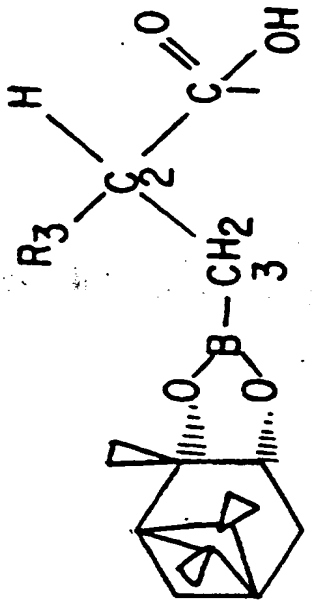


XLII



XLIII

FIG 14B



XLIV

PCT

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(74) Agent: COOPER, Iver, P.; Browdy and Neimark, 419 Seventh Street, Ste. 300, N.W., Washington, DC 20004 (US).

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(57) Abstract

Novel small proteins which bind elastase or cathepsin G have been identified. These are useful as inhibitors of excessive elastase or cathepsin G activity in patients.

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INTERNATIONAL SEARCH REPORT

International Application No PCT/US 92/01501

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/15; C07K5/02; A61K37/64

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C07K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
A	EP,A,0 307 592 (BAYER AG) 22 March 1989 cited in the application see page 22, line 10 - line 20 see page 2, line 49 - page 3, line 38; claims 1-19	1-2,4-6, 12-13
A	BIOL. CHEM. HOPPE-SEYLER, VOL. 371, SUPPL. May 1990, BERLIN, FRG pages 43 - 52 T. BRINKMANN & H. TSCHESCHE 'Design of an Aprotinin Variant with Inhibitory Activity against Chymotrypsin and Cathepsin G by Recombinant DNA Technology' cited in the application see abstract	2,4-6

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
28 OCTOBER 1992	04. 11. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	THIELE U.H.-C.H.

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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	EP,A,0 401 508 (BAYER AG) 12 December 1990 see abstract; claims 1-8,10	3-6, 12-13
A	WO,A,9 002 809 (PROTEIN ENGINEERING CORPORATION) 22 March 1990 see example 1	1-6
A	EP,A,0 195 212 (MERRELL DOW PHARMACEUTICALS INC.) 24 September 1986 see page 8, line 11 - line 15; claims 1-7 see page 12, line 16 - line 20	7,12-13

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